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STUDIES ON RESPIRATORY DISEASES

XXV. THE INFLUENCE OF CERTAIN ELECTROLYTES AND NONELECTROLYTES ON THE BILE SOLUBILITY OF PNEUMOCOCCI *

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Since the discovery of the bile solubility of pneumococci by Neufeld, a large number of studies have been reported bearing chiefly on the technic for conducting the test and on its value in the detection, identification and estimation of the virulence of pneumococci. Almost no attention has been given to an analysis of the mechanism of the phenomenon. The studies reported here were undertaken with the hope that they might cast some light on the dynamics of the reaction. For the approach to the problem we have borrowed from methods which have been used widely in those fields of general physiology treating of the semipermeability of protoplasm to the menstruum which bathes it. Our procedure has been to study the influence of certain reagents on the solubility of pneumococci and of other bacteria by bile.

For the purposes of our experiments, recently isolated and stock strains of pneumococci were grown on blood agar slants containing 5% sheep blood. In each case, the growth on the slanted surface was washed off with glucose broth (veal infusion broth containing 0.25% glucose) and incubated at 37 C. for 20 minutes. A portion of this emulsion was transferred to a flask containing 150 c c. of glucose broth (P_H 7.8) and incubated at 37 C. for 24 hours. Unless unwashed suspensions are specified, the bacteria in the fluid cultures were washed free from the constituents of the culture medium by being centrifugalized, taken up in water, reprecipitated and the process repeated from 3 to 6 times. Contrary to the belief which is widely held, pneumococci which have been washed and resuspended in water undergo no appreciable autolysis at temperatures between 0 and 20 C. in the few hours elapsing in the preparation of a bile solubility experiment. This finding makes possible the use of suspensions in water and eliminates the uncertain influences of the 0.8% NaCl which is commonly (and erroneously) used as though it were an indifferent "physiological" medium. The reagents whose influence on bile solubility were tested were added to the bacterial suspensions in the concentrations and volumes indicated in the tables so as to avoid relative changes in the concentration of bacteria. Usually, the procedure followed was to add 0.5 c c. of reagent solution to 0.5 c c. of bacterial suspension.

Preliminary experiments confirmed the findings reported from this laboratory by Kelley and Gussin[1] (1) to the effect that the acidity which is developed in

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

[1] Jour. Infect. Dis., 1924, 35, p. 327.

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glucose broth cultures of pneumococci does not interfere with the solution of washed or unwashed pneumococci by bile, and (2) that stored, untreated, whole ox bile is a more effective solvent for pneumococci than fresh or fresh, autoclaved bile. We have therefore used stored bile in the experiments reported here.

EXPERIMENTAL FINDINGS

From preliminary experiments, it appeared that with some strains after washing with water the pneumococci are no longer soluble in bile. Hence the experiment reported in table 1 was conducted to determine the influence of NaCl and KCl on the bile solubility of a stock strain of pneumococcus type 3 which shows such a change in solubility as well as on strains which are soluble in distilled water suspensions.[2]

TABLE 1

THE INFLUENCE OF NaCl AND KCl ON THE BILE SOLUBILITY OF PNEUMOCOCCUS (TYPE 3)

0.5 c c. salt solution + 0.5 c c. washed pneumococci incubated at 37 C. for 20 min.; no change. 0.25 c c. bile added; incubated at 37 C. for 20 min.

Salt Solution Used, %	Bile Solubility with	
	NaCl	KCl
0	+ (o)*	+ (o)*
0.01	0	0
0.1	0	0
0.25	0	0
0.5	0	0
0.75	0	0
1.0	0	0
2.5	0	0
5.0	+	+
10.0	++	++

* The (o) is introduced here to indicate that an occasional preparation fails to show lysis of aqueous suspensions lacking salt.

The experiment reported in table 1 was repeated, and similar results were obtained. Good bacteriolysis occurred in the mixtures containing NaCl or KCl in final concentrations of 0, 2 and 4% (0.5 c c. of 5 and 10% solutions + 0.5 c c. bacteria + 0.25 c c. bile). The results with the two salts were indistinguishable. A similar effect on the bile solubility of 24-hour glucose broth cultures by these salts was also observed in another series of experiments, but the effect appeared only after incubation for some hours. These experiments indicate that the dissolution of washed pneumococci by bile usually occurs with distilled water suspensions to which no salt is added; that small concentrations of NaCl and KCl inhibit and higher concentrations do not inhibit the solution.

[2] The strains which fail to show lysis with bile in distilled water are the exception, not the rule, and are only occasionally encountered.

A similar set of experiments were conducted with two strains of pneumococcus type 3 obtained from the laboratories of the N. Y. State Department of Health to determine the influence of $CaCl_2$ and $BaCl_2$ on bile solubility.

From an examination of the data in table 2 it appears that calcium and barium chlorides inhibit the bile solubility of washed pneumococci; that the extent of the inhibition increases with increasing concentration of the salt; and that $CaCl_2$ is more inhibitive than $BaCl_2$ when the two salts are used in the same percentage concentration. Thus, with culture *a* $CaCl_2$ completely inhibits bile solubility in a final [3] concentration of 0.2% and $BaCl_2$ inhibits in 1.0% concentration. Similarly, with culture *b* the inhibiting concentrations are 0.2% for $CaCl_2$ and 0.4% for $BaCl_2$.

TABLE 2

THE INFLUENCE OF $CaCl_2$ AND $BaCl_2$ ON THE BILE SOLUBILITY OF PNEUMOCOCCI (TYPE 3)

0.5 c c. salt solution + 0.5 c c. washed pneumococci; incubated at 37 C. for 20 min.; no change. 0.25 c c. bile added; incubated at 37 C. for 20 min.

Salt Solution Used, %	Bile Solubility			
	Culture *a*		Culture *b*	
	$CaCl_2$	$BaCl_2$	$CaCl_2$	$BaCl_2$
0	+++	+++	++	+++
0.01	++	++	++	++
0.1	+	+	+	++
0.25	±	+	±	++
0.5	0	+	0	+
0.75	0	±	0	+
1.0	0	±	0	0
2.5	0	0	0	0
5.0	0	0	0	0
10.0	0	0	0	0

If the difference between the molecular weights of the two salts be taken into consideration ($CaCl_2 = 111$; $BaCl_2 = 208$), the ratios between inhibiting concentrations for the two salts are approximately halved and are brought closer together, i. e., for culture *a*, 0.2% for $CaCl_2$ and 0.5% for $BaCl_2$; for culture *b*, 0.2% for $CaCl_2$ and 0.2% for $BaCl_2$. Hence, it may be considered that the inhibitory effects of these two salts are almost identical.

In experiments on the influence of $CaCl_2$ and $BaCl_2$ on bile solubility, an insoluble precipitate is sometimes formed when the bile is added to the salt solution. The formation of this precipitate can be avoided by using fresh bile or by neutralizing the alkalinity of old bile.

[3] Final concentration refers to the concentration calculated by making allowance for the dilution of the salt solution by the volumes of the bile and of the aqueous suspension of bacteria.

The results of experiments with type 3 pneumococci presented in tables 1 and 2 appear to indicate that all the salts tested inhibit bile solution of the bacteria when they are added in certain concentrations. A significant difference appears, however, between the salts with monovalent and those with divalent cations. The former are inhibitory only in low concentrations; the latter are inhibitory only in high concentrations. The general validity of these findings was confirmed for ammonium and lithium chlorides with cultures of types 1, 2, 3 and 4 and for calcium and barium chlorides with strains of types 1 and 2.

From inspection of table 3 it appears that the strains of pneumococci used differ in their susceptibilities to solution by bile in the presence of salts; and that in equipercentage concentrations NH_4Cl is more effective

TABLE 3

THE INFLUENCE OF AMMONIUM AND LITHIUM CHLORIDES ON THE BILE SOLUBILITY OF PNEUMOCOCCI (TYPES 1, 2, 3 AND 4)

0.5 c c. salt solution + 0.5 c c. washed pneumococci incubated at 37 C. for 20 min.; no change. 0.5 c c. bile added; incubated at 37 C. for 20 min.

Salt Solution, %	Bile Solubility							
	NH_4Cl				LiCl			
	Type 1	Type 2	Type 3	Type 4	Type 1	Type 2	Type 3	Type 4
0	+	+	+	+	+	+	+	+
0.01	0	0	0	0	0	0	0	0
0.1	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0
0.5	±	±	0	0	0	0	0	0
0.75	±	±	0	0	0	0	0	0
1.0	+	±	±	±	±	0	0	0
2.5	+	+	+	±	±	±	±	0
5.0	+	+	+	±	+	+	+	0
10.0	+	+	+	+	+	+	+	+

than LiCl in enhancing bile solution. As the molecular weight of NH_4Cl (53.5) is greater than that of LiCl (42.4), the observed differences between the salts are slightly increased if corrected for the gram-molecular ratio of 5 : 4.

From table 4 it appears that for pneumococci of types 1 and 2—as has been indicated for type 3 in table 2—$CaCl_2$ and $BaCl_2$ inhibit bile solubility if the concentration of salt be raised sufficiently. Again the differences in the percentage of salt which suffices to give complete inhibition is higher for the barium than for the calcium salt. This difference becomes nearly negligible if the concentrations are reduced to an equimolar basis.

On attempting to expand the series of salts with divalent cations in further experiments, it was found that the presence of inert precipitates

formed by the salts with bile rendered solution of the pneumococci indeterminate. Hence, the succeeding experiments were devoted to studies with salts varying in their anions.

It has long been known that pneumococci, as well as other micro-organisms, are dissolved by sodium hydroxide. Kruse [4] showed that gram-negative bacteria are somewhat more susceptible to the cytolytic properties of sodium hydroxide solutions than gram-positive organisms. More recently, Homer Smith [5] has reinvestigated this reaction in the course of an extensive study on the biochemical properties of bacteria. He removed the growth of bacteria from 24-hour agar slant cultures with 0.5% NaCl solution and added 1 drop of emulsion to 1 c c. portions

TABLE 4

THE INFLUENCE OF CALCIUM AND BARIUM CHLORIDES ON THE BILE SOLUBILITY OF PNEUMOCOCCI (TYPES 1 AND 2)

0.5 c c. salt solution + 0.5 c c. washed pneumococci; incubated at 37 C. for 20 min.; no change. 0.5 c c. bile added; incubated at 37 C. for 20 min.

Salt Solution %	Bile Solubility			
	$CaCl_2$		$BaCl_2$	
	Type 1	Type 2	Type 1	Type 2
0	+	+	+	+
0.01	+	+	+	+
0.1	+	+	+	+
0.25	0	+	+	+
0.5	0	+	+	+
0.75	0	±	±	±
1.0	0	0	±	±
2.5	0	0	0	0
5.0	0	0	0	0
10.0	0	0	0	0

of NaCl solutions of NaOH of various concentrations and observed the cytolytic effects after 2 hours. He found (if our interpretation of his charted data is correct) that "Dip. pneumoniae" is dissolved by concentrations of NaOH up to 10%, but not by more dilute solutions. It has been supposed that cytolysis by NaOH is a function of the C_{OH^-}.

We have found that of a series of salts having Na^+ as a common cation and differing in their anions, only NaOH and Na_3PO_4 are cytolytic toward pneumococci. In no case, however, was cytolysis observed when the washed bacteria were prepared from cultures incubated less than 24 hours. Na_2SO_4, Na_2HPO_4, NaH_2PO_4, and $NaNO_3$ were without evident cytolytic action in the concentrations studied.

[4] München. med. Wchnschr., 1909, 56, p. 897.
[5] Am. Jour. Hyg., 1922, 2, p. 607.

Other Na salts studied were soon eliminated from the anion series because they formed confusing insoluble precipitates with the bacteria. The data are presented in table 5.

It appears from table 5 that NaOH was cytolytic to washed type 1 pneumococci in concentrations as low as about 0.3% (0.4 c c. of 1% NaOH + 1 c c. washed bacteria). Tribasic sodium phosphate was cytolytic only in the two highest concentrations used—1.4% and 2.8%. These results would imply greater susceptibility of washed than of

TABLE 5

THE CYTOLYSIS OF TYPE 1 PNEUMOCOCCI BY CERTAIN SODIUM SALTS

0.4 c c. salt solution + 1 c c. washed pneumococci; incubated at 37 C. for 20 min. and 2 hrs.

Salt Solution, %	Cytolysis with					
	NaOH	Na_3PO_4	Na_2HPO_4	NaH_2PO_4	$NaNO_3$	Na_2SO_4
0.001	0	0	0	0	0	0
0.01	0	0	0	0	0	0
0.1	0	0	0	0	0	0
1.0	+	0	0	0	0	0
5.0	+	+	0	0	0	0
10.0	+	+	0	0	0	0

TABLE 6

THE INFLUENCE OF PEPTONE, GELATIN AND OVALBUMIN ON THE BILE SOLUBILITY OF PNEUMOCOCCI (TYPES 1, 2 AND 3)

0.5 c c. of solution of test substance + 0.5 c c. of washed pneumococci; incubated at 37 C. for 20 min.; no change. 0.5 c c. bile added; incubated at 37 C. for 20 min.

Solution of Test Substance, %	Bile Solubility with								
	Peptone			Gelatin			Ovalbumin		
	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
0	+	+	+	+	+	+	+	+	+
0.01	+	0	0	+	+	+	+	+	+
0.1	+	0	0	+	0	0	0	0	0
0.25	0	0	0	+	0	0	0	0	0
0.5–10.0	0	0	0	0	0	0	0	0	0

unwashed cells to the cytolytic action of the electrolyte and suggest that the cytolysis is justly considered a function of the hydroxyl ion concentration.

The greater susceptibility of washed than of unwashed pneumococci to cytolytic agents indicates the possibility that proteins, peptones and other reagents similar to substances present in bacterial cultures will be found to inhibit the dissolution of pneumococci by bile. In table 6 we present the results of experiments designed to verify this point.

It appears from table 6 that all 3 of the nitrogenous substances tested —peptone, gelatin and ovalbumin—inhibit the cytolytic action of bile for pneumococci of the 3 fixed types. Ovalbumin appears to exert its effective inhibitory action in lowest and gelatin in highest concentration. The dissolution of pneumococci appears to be less easily inhibited if they are of type 1 than of either of the other types.

SUMMARY

Washed suspensions of penumococci in distilled water are usually bile soluble. An occasional preparation is refractory to the solvent action of the bile.

Chlorides with monovalent cations (Na, K, NH_4, Li) in relatively low concentrations inhibit solution of washed pneumococci by bile.

In higher concentrations these chlorides do not inhibit and may accelerate the dissolution of the bacteria.

Chlorides with divalent cations (Ca, Ba) behave differently. They are found to inhibit bile solution of pneumococci more effectively in high than in low concentrations.

Of the anion series tested, NaOH and Na_3PO_4 are cytolytic to pneumococci; Na_2HPO_4, NaH_2PO_4, NA_2SO_4 and $NaNO_3$ are not cytolytic.

Cytolysis by NaOH and Na_3PO_4 appears to be a function of hydroxyl ion concentration.

Peptone, gelatin and ovalbumin appear to inhibit cytolysis by bile in the same manner as $CaCl_2$ and $BaCl_2$. The inhibitory action increases with concentration.

The differences in the behavior of chlorides of monovalent and of divalent cations recorded here appear to be in harmony with the usual findings in general physiology, whether effects on animals and animal tissues, on chlorophyll-bearing plants, bacteria or on nonliving substances are studied. These general relations have been discussed at length by Falk.[6] Their bearing on the mechanism of bacteriolysis will be treated in a later publication from this laboratory.

[6] Bibliographic Review, Abst. Bacteriol., 1923, 7, p. 33.

STUDIES ON RESPIRATORY DISEASES

XXVI. THE LYSIS OF PNEUMOCOCCI BY SODIUM OLEATE *

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In recent years there has been a growing tendency to prepare antigenic material from pneumococci for immunologic work by dissolving the organisms with bile. The complex and variable constitution of bile renders its use for this purpose a procedure of uncertain significance. There has also been evident a tendency on the part of various students of pneumococcus pneumonia to use the bile solubility reaction as a test for the relative virulence of a strain as well as for its identification. This has grown out of the common observation that avirulent strains of pneumococci are frequently found to be insoluble in bile. It is not unlikely that variations in the findings of studies on the relations between bile solubility and virulence of pneumococci are, in a measure at least, determined by variable characteristics of the bile used in the test.

From considerations on these problems we were persuaded that it would be desirable, if it is possible, to substitute for bile a reagent of simpler and more uniform composition in studies on nonimmune bacteriolytic reactions. From an a priori analysis of the mechanism of bacteriolysis, we decided to study the lytic properties of substances which reduce the surface tension of water and the interfacial tension of colloidal suspensions in water. The soaps—particularly sodium oleate—and certain other substances have been studied.

It has long been known that it is possible to extract from normal tissues material which is bactericidal to organisms of various species, that the active constituents in the extract are probably higher unsaturated fatty acids or their soaps, and that these substances are often hemolytic.[1] Lamar discovered that although antipneumococcus serum possesses in itself neither bactericidal nor bacteriolytic properties, it comes to exert both of these actions when it acts in conjunction with certain other substances. He studied the activities of soaps of oleic acid with Na, NH_4 and neurin, and found that the sodium soap is the most active. It dissolves pneumococci, virulent for white mice, in broth cultures when

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

[1] See review by Flexner in the paper by Lamar: Jour. Exper. Med., 1911, 13, p. 1.

present in concentrations between 1 and 0.5%. In concentrations insufficient to produce their lysis or to affect their viability or morphology, sodium oleate renders pneumococci more readily lysed by serum or by their own autolytic enzymes. The lytic action of sodium oleate, he found, is inhibited by proteins; the inhibitory action of proteins can be neutralized with boric acid; and mixtures of there reagents with serum will prevent infections in animals into which pneumococci have been injected. In his second paper, Lamar reported that certain other soaps are bacteriolytic and hemolytic and that two (sodium linoleate and sodium linolenate) are respectively about 4 and 6 times as destructive as sodium oleate. Later, Lamar [2] found that the linoleate and linolenate are not as effective as the oleate in preventing pneumococcus infections.

TABLE 1

THE BACTERIOLYTIC ACTION OF BILE, SODIUM OLEATE AND SAPONIN ON PNEUMOCOCCI (TYPES 1, 2 AND 3)

0.5 c c. of washed pneumococci + 0.5 c c. of test solution

Final Concentration of Test Substance, %	Bacteriolysis with		
	Bile Types 1, 2, 3	Sodium Oleate Types 1, 2, 3	Saponin Types 1, 2, 3
0.5	0	0	0
1.25	0	+	0
2.5	+	+	0
5.0	+	+	0

EXPERIMENTAL FINDINGS

To avoid the interfering action of proteins, salts and other substances in bacteriolytic reactions, we have used washed suspensions of bacteria in distilled water in the experiments reported here (Falk and Yang [3]). In table 1 we present the results of an experiment designed to determine the comparative lytic properties of bile, sodium oleate and saponin for pneumococci of the fixed types. Whole, stored bile, Na.oleate solutions in water and saponin solutions in 0.85% NaCl were used. In preliminary tests the saponin was found to be actively hemolytic.

It is clear from table 1 that in the concentrations over 1.25% bile is bacteriolytic; that sodium oleate is bacteriolytic in the lowest concentration used (0.5%); and that saponin does not effect bacteriolysis of pneumococci in concentrations between 0.5% and 10%. In subsequent experiments we undertook to ascertain the generality of these findings. To this end, bacteriolytic tests were conducted with whole, stored bile

[2] Ibid., 1911, 14, p. 256.
[3] Jour. Infect. Dis., 1926, 38, p. 1.

and with 10% sodium oleate solutions on a series of pneumococcus strains available in this laboratory. The results of a typical experiment are cited in table 2.

Of the 16 strains of pneumococci used, 6 showed both bile and oleate solubility. None of the other strains was dissolved by either reagent. No differences were observed between the two test substances. Both failed to dissolve the hemolytic streptococcus used as a control. It appears, further, from table 2 that the results obtained with the several strains were independent of their serologic types. The 3 strains designated "N.Y." had been obtained from the laboratories of the N. Y. State Dept. of Health; the strains "Rock" had come from the Rockefeller Institute for Medical Research; the others had been isolated some months earlier from cases of acute lobar pneumonia in the Cook County Hospital of Chicago and were of the series described by Falk, Gussin and Jacobson.[4]

TABLE 2

BACTERIOLYSIS OF PNEUMOCOCCI BY BILE AND SODIUM OLEATE

1 c c. of washed pneumococci in water + 0.5 c c. of bile or 10% sodium oleate solution in water

Strain of Pneumococcus	Bacteriolysis with	
	Bile	Sodium Oleate
N. Y.—types 1, 2, 3	+	+
Rock.—types 1, 2, 3	+	+
H 116, M 162, G 71—type 1	0	0
M 173, S 34, B 170—type 2	0	0
Wash., C 139, P 35, A 11—type 4	0	0
Hemolytic strep. (control)	0	0

Following the experiments of Lamar to which we have referred, we undertook to determine the extent of inhibition of bile and oleate bacteriolysis by nitrogenous substances. We have also studied the inhibitory action of sodium chloride. For these experiments, 24-hour cultures of pneumococci in glucose veal infusion broth were tested for bile and oleate solubility. They were found to be soluble. The bacteria were then washed 4 times with water by repeated centrifugalization and resuspension. After the last washing the bacteria were divided into 3 portions. These were resuspended, respectively, in distilled water, in 0.85% solution of NaCl in distilled water, and in the culture fluid supernatant from which the bacteria had been separated. They were then tested with bile and Na oleate (table 3).

It is clear from table 3 that bile and oleate solution of pneumococci were not obtained in mixtures in which these reagents were present in

[4] Jour. Inf. Dis., 1925, 37, p. 481.

final concentrations below 2.5%; that in final concentration of 5% (10% solution + equal volume of bacterial suspension) sodium oleate fails to dissolve pneumococci which are dissolved by bile in the same concentrations; that with washed suspensions in distilled water both bile and oleate were effective solvents in 2.5% final concentrations; that 0.85% NaCl inhibits oleate solution completely and bile solution partly; and that the menstruum of the broth cultures inhibits the solvent action of both test substances acting on the washed organisms to give the same results as were obtained with the untreated, original broth cultures. Whenever there were any differences among pneumococci of the several types they were confirmatory of the findings reported by Falk and Yang [3]; that the solution is less easily inhibited with type 1 than with

TABLE 3

THE INHIBITION OF BILE AND SODIUM OLEATE SOLUTION OF PNEUMOCOCCI (TYPES 1, 2 AND 3) BY BROTH AND BY SODIUM CHLORIDE

0.4 c c. of bacterial suspension + 0.4 c c. of solvent

Solvents, %	Original Broth Cultures, Types 1, 2, 3	Washed Suspensions in								
		Water			0.85% NaCl			Broth Supernatant		
		Types			Types			Types		
		1	2	3	1	2	3	1	2	3
Bile—0.001, 0.01, 0.1 and 1.0	0	0	0	0	0	0	0	0	0	0
5.0	0	+	+	+	+	+	+	0	0	0
10.0	+	+	+	+	+	±	±	+	+	±
Sodium oleate—0.001, 0.01, 0.1 and 1.0	0	0	0	0	0	0	0	0	0	0
5.0	0	+	+	0	0	0	0	0	0	0
10.0	0	+	+	+	0	0	0	0	0	0

types 2 and 3 organisms. It is significant to remark that the inhibition of pneumococcus dissolution by broth confirms the findings of Lamar and extends them to include bile as well as oleate solution; and that the inhibitory action of 0.85% NaCl solution on oleate solution indicates that inhibition is not limited to the action of solutions containing nitrogenous constituents only. Indeed, the implication is that inhibition of the effectiveness of oleate as a solvent agency for pneumococci may be a characteristic of a variety of reagents.

In the preceding publication on the dissolution of pneumococci, we reported on the solvent action of sodium hydroxide and of tribasic sodium phosphate as well as of bile. In the experiments typified by the data in table 4 we used these reagents as well as sodium oleate to determine their efficacy in dissolving bacteria other than pneumococci. For

these comparative experiments we used 6 strains of pneumococci which show bile solubility regularly, 5 strains of hemolytic streptococcus, one each of Str. viridans, Str. lacticus, Neisseria catarrhalis, Sarcina lutea and Staphylococcus albus and 4 strains of Staph. aureus which were available in this laboratory.

It is clear from table 4 that with the concentrations of reagents used the solvent properties of sodium oleate, hydroxide and phosphate are as specific for the pneumococcus as those of bile. None of the bacteria, other than the pneumococci, showed any bacteriolytic effects of the test substances. We may therefore conclude that the bacteriolysis caused by these reagents is as definitely determined by specific characteristics of these bacteria as by the properties of the reagents.

TABLE 4

The Dissolution of Bacteria of Several Genera and Species by Bile, Sodium Oleate, Sodium Hydroxide and Sodium Phosphate

1 c c. of washed bacteria + 0.5 c c. of 10% solution of test substances

Strain	Solution with			
	Bile	Na Oleate	NaOH	Na_3PO_4
Pneumococci: N. Y., 1, 2, 3. Rock., 1, 2, 3	+	+	+	+
Str. hemol.: C. D. H., 221, canine, 27.2; Str. viridans, Str. lacticus, N. catarrhalis, Sarc. lutea, Staph. albus, and Staph. aureus: 43, T 20, C 17, and Nors.	0	0	0	0

SUMMARY

In concentrations of 1.25% and higher, pure solutions of sodium oleate in water are bacteriolytic to washed suspensions of pneumococci of types 1, 2 and 3.

Saponin in concentrations of 0.5%-10% in 0.85% NaCl does not dissolve pneumococci.

In the concentrations in which it is active, sodium oleate dissolves only pneumococci of those strains which are also dissolved by bile.

Sodium oleate, in effective concentrations in water, dissolves washed pneumococci resuspended in distilled water. It does not dissolve washed pneumococci which have been resuspended in 0.85% NaCl solution or in the supernatent of the culture fluid from which they had been separated by centrifugalization.

In the concentrations used in the experiments reported, the bacteriolysis of washed pneumococci by sodium oleate, sodium hydroxide and sodium phosphate (tribasic) is as specific as bile solubility. Suspen-

sions of bile insoluble strains of pneumococci, of strains of hemolytic streptococcus, Str. viridans, Str. lacticus, Neisseria catarrhalis, Sarcina lutea, Staphylococcus albus and of Staph. aureus are not dissolved by these reagents.

It appears that the dissolution of pneumococci by bile, sodium oleate, sodium hydroxide and sodium phosphate is determined as definitely by the specific characteristics of these bacteria as by the properties of the solvent agent.

Because of the chemically pure state in which it can be prepared and its certain composition, sodium oleate may serve useful purposes in bacteriolytic experiments with pneumococci in which bile of uncertain and variable composition is now generally used.

The bacteriolytic action of soaps—as typified by sodium oleate—is specific to pneumococci among the bacteria studied. Hence, we are of the opinion that the functional rôle of these substances is not of general significance in bacteriolytic phenomena of pathology.

THE MECHANISM OF THE RUSSELL DOUBLE SUGAR TUBE

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In the fall of 1923, we became interested in the mechanism of the Russell double sugar tube through some casual observations of the reactions under anaerobic conditions. A series of experiments was planned and partly carried out when the paper of Wheeler [1] dealing with the same subject came to our notice. In most points, the conclusions reached by Wheeler were in accord with the results of our experiments, but in certain details we fail to agree with him. We have approached the matter somewhat differently, and as we have used not only typhoid bacilli in the work but also representative members of the related subgroups of organisms, we feel that our conclusions are perhaps worth a brief statement.

The Russell medium consists of a nutrient agar base to which is added 0.1% of dextrose and 1% of lactose, plus a suitable indicator. Relatively large amounts are placed in each tube and cooled in such a position as to give a rather short slant and a deep butt. It is inoculated both by spreading material on the slant surface and by stab into the butt. Grown in the air, lactose fermenting organisms show acid reactions in both slant and butt. Those fermenting dextrose but not lactose show acid butts and alkaline slants. The ability to form gas from these sugars is shown by the development of bubbles along the line of the stab.

It will be recalled that in his original description of this medium, Russell [2] stated the opinion that the characteristic reaction was due to the differences of oxygen tension existing on the slant and in the butt of the tube, while Nichols,[3] and Nichols and Wood [4] believe that the alkalinity of the slant when typhoid is grown is due to the diffusion out of the medium of volatile acids, largely carbonic, and the retention of the simultaneously produced alkaline products of fermentation. Wheeler, on the other hand, presented evidence leading him to believe that the diffusion of volatile acids played a small part in the final reaction of the slant portion of the tube, but that the alkaline reaction was the result of the prepon-

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[1] Jour. Infect. Dis., 1924, 34, p. 13.
[2] Jour. Med. Res., 1911, 25, p. 217.
[3] Jour. Infect. Dis., 1921, 29, p. 82.
[4] Ibid., 1922, 30, p. 320.

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derance of alkaline fermentation for which he believed oxygen to be necessary. If the amount of acid produced from the fermentable carbohydrate present was insufficient to neutralize the alkali produced, the slant remained alkaline. If a larger amount of fermentable sugar was present, as is the case in the Russell tube when inoculated with colon bacilli, the quantity of acid was in excess and the slant becomes acid. Wheeler showed that acid fermentation of dextrose by the typhoid bacillus goes on under anaerobic as well as aerobic conditions, that the fermentation of 0.1% of dextrose is complete in 24 hours, and that when a Russell tube is coupled with a tube containing alkaline pyrogallol, in a closed system, producing anaerobic conditions and at the same time providing for the rapid removal from the contained gases of any volatile acid products of bacterial growth, the slant of the tube became acid in the same way as when the tube was merely stoppered. This last observation directly contradicts the statement of Nichols as to the results of the same experiment. Wheeler finally concluded that the ultimate alkaline reaction seen in the butt of the tube was due, not to diffusion of alkaline substances from the slant, but to the later production of alkali in the butt itself, owing to penetration of oxygen.

Nichols' suggestion that the phenol-red indicator is the most satisfactory for use in the Russell tube, we strongly endorse, because it shows changes in the hydrogen ion concentration in both directions. While the Andrade indicator [5] is much more satisfactory than the original litmus, it not only fails to indicate an alkaline change, but also is decidedly less sensitive to acid changes than is phenol red. For this reason, while we have used both indicators extensively in the course of this work, our conclusions are all based on the results shown by the phenol red tubes; those with Andrade are not consistent within the limitations of the indicator. For growth under anaerobic conditions we used the Brown modification of the McIntosh and Fildes [6] jar, with a tube of glucose broth colored by methylene blue as an indicator of anaerobiosis.

Our initial observation was that when typhoid bacilli were grown anaerobically in such a jar, the slant as well as the butt of Russell tubes became strongly acid. If the explanation of Nichols and Wood were correct, this observation would imply that the tubes produced sufficient carbonic acid to render the gaseous contents of the jar so acid as to change the reaction of the slants.

[5] Krumwiede, Pratt and McWilliams: Ibid., 1916, 18, p. 1.
[6] Lancet, 1916, 1, p. 768.

Exper. 1.—Two Russell tubes inoculated with typhoid bacilli, one with phenol red and one with Andrade indicator, were placed in the anaerobic jar, together with 2 uninoculated tubes, and the oxygen was exhausted. After 20 hours' incubation at 37 C., the inoculated tubes were strongly acid, both slant and butt, while the control tubes remained unchanged. The jar used in these experiments had a capacity of about 250 cubic inches.

Exper. 2.—Two Russell tubes inoculated with typhoid bacilli, one with phenol red and one with Andrade indicator, were placed in the anaerobic jar. A 4 inch Stender dish containing a concentrated solution of KOH was also placed in the jar, and the oxygen was exhausted. After 20 hours' incubation, the inoculated tubes were strongly acid, slant and butt, while control uninoculated tubes remained unchanged.

Exper. 3.—A typhoid-inoculated Russell tube with phenol red indicator was placed in a 100 c c. cylinder containing 20 c c. of alkaline pyrogallol; the cylinder was then stoppered and sealed with paraffin. After 20 hours' incubation, the tube was strongly acid in both slant and butt.

These results were interpreted as indicating that the typhoid organism fermented carbohydrate under anaerobic conditions, and that the difference in the reaction of the slant and butt of the Russell tube when incubated in the air was related to the difference of oxygen tension in the two parts of the tube, as suggested by Russell. We next tried the same experiment with other members of the colon-typhoid group of bacteria, using several different strains of typhoid, dysentery—Shiga and Flexner—paratyphoids A and B, Aertrycke, Gaertner and colon bacilli. The results with these organisms confirmed the findings with typhoid. The members of the paratyphoid and colon groups produced gas in the butts the same as when grown aerobically. All the typhoid, dysentery and colon strains examined produced acid slants both with Andrade and with phenol red indicators. Only in the paratyphoid group was there any departure from the general rule. The members of this group all failed to show acid on the slants of tubes with Andrade indicator, with the exception of the strains of paratyphoid A. These strains consistently showed a faint pink color with Andrade indicator, distinctly less intense, however, than the color shown by the typhoid, dysentery and colon strains. This difference between the members of the Salmonella group and the others was shown also by the phenol red tubes, although here it was evident that acid was produced in all the tubes although the color change was decidedly less than was seen with typhoid, dysentery and colon strains. The paratyphoid A tubes again were intermediate in color between the B group and the rest of the set. Incubation for 48 hours produced no change in these relations.

An interesting observation was made at this time which proved to be the key to the rest of our work. It was noted that the phenol red tubes

inoculated with nonlactose fermenters, incubated for 20 hours anaerobically and removed from the jar for examination and record, tended rapidly to revert to the appearance of the air-grown tubes. The slants often began to turn red along the margins within 10 minutes, and were usually indistinguishable from the air-grown controls in an hour or two.

Wheeler attributed the ultimate alkaline reversion of the butt of the tube to continued production of alkali by the growing bacteria, and his experience with anaerobic tubes when exposed to air after incubation appears to be at variance with ours. Our observation was frequently repeated, however, with constant results, and the great rapidity of the alkaline reversion seemed to us incompatible with the supposition that the change was due to the continued activities of the organisms concerned. Accordingly, the following experiments were carried out:

Exper. 4.—A phenol red Russell tube was inoculated with typhoid bacilli, stoppered, sealed and incubated over night. The tube was then immersed in water at a temperature of 80 C. for 1 hour, the stopper being held firmly in position. The tube was then taken from the water bath, the stopper removed and material from the slant inoculated into a fresh tube. The original tube was then exposed to the air for 24 hours. At the end of the first period of incubation, both slant and butt were strongly acid. Heating the closed tube produced no further change. On exposure to air, the slant began to turn red within 15 minutes, and within 4 hours the tube was indistinguishable from one grown in the air. The transplant made after the heating failed to show any growth.

Exper. 5.—Two phenol red Russell tubes were inoculated with typhoid bacilli. One had been incubated for 20 hours anaerobically and the other had been freshly inoculated. Both were placed in a large beaker containing ice and salt, and chilled to below the freezing point. They were then placed in the icebox at 6 C. and examined after 1 hour and after 24 hours. A 20-hour anaerobic Russell tube with typhoid bacilli was kept in the air at room temperature as a further control. The chilled tube reverted to alkaline in the slant almost as rapidly as the tube at room temperature, showing well marked red coloration along the edges after 30 minutes, while the reversion was complete after 24 hours. One-half the butt was alkaline in 48 hours. The freshly-inoculated tube placed on the ice showed no change until after 72 hours, when a slight growth appeared without change of reaction.

These experiments appear to show that the alkaline reversion was not dependent on continued activity of the bacteria. They were confirmed by the following test.

Exper. 6.—A phenol red Russell tube was inoculated on the slant only with typhoid bacilli and incubated for 20 hours anaerobically. The growth was then washed from the surface with a 5% phenol solution in a phosphate buffer having a P_H value of 7.4. After an hour in the air an alkaline change began to be visible, and after 24 hours the entire slant was alkaline.

These observations led us to believe that there was produced in the anaerobic cultures some substance, not itself alkaline in reaction, which was rapidly oxidized on exposure to air into a substance giving an alkaline reaction. In order to test the correctness of this supposition, the following experiments were performed.

Exper. 7.—A phenol red Russell tube was inoculated with typhoid bacilli and stoppered with a double perforated rubber stopper through which glass tubes were passed, an inlet tube reaching near the lower end of the slant and an outlet tube ending just below the stopper. The free ends of these tubes were drawn out and sealed in the flame. After 24 hours' incubation, both slant and butt were strongly acid. The tube was then connected with an oxygen tank and the gas run through rapidly for several minutes, and the tube again closed with the oxygen under some pressure. The slant became strongly alkaline within 1 hour, but on standing for 24 hours again became acid.

Exper. 8.—This was the same as exper. 7 except that instead of passing oxygen through the tube, it was connected with a hydrogen generator, the gas passed through for several minutes, and the tube closed. The tube in this instance showed no change.

Exper. 9.—Ten c c. of phenol red, plain broth, P_H 7.4 was inoculated with typhoid bacilli and with other members of the group in an ordinary test tube. The tube was then stoppered with a double perforated rubber stopper carrying 2 glass tubes, one of which reached to the bottom of the tube, while the other ended just below the stopper. The distal ends of these tubes were drawn out and sealed. The entire apparatus was sterilized in the autoclave before inoculation. The inoculated tube was incubated for 48 hours and its P_H value noted. It was then connected with a hydrogen generator in such a way that when the sealed ends of the tubes were broken, hydrogen could be bubbled through the tubes. The hydrogen was passed through 2 wash bottles containg alkaline pyrogallol and tested for freedom from oxygen by its ability to burn without explosion. The gas was passed through the tube for ten minutes and the P_H value again noted. The hydrogen tube was then removed, the apparatus connected with the suction pump, and air drawn through the tube for 10 minutes. The air in the laboratory was tested in a control experiment and found to be without effect on the reaction after a period of an hour. Preliminary experiments showed that after 10 minutes' treatment with hydrogen or with air, no further change occurred.

This experiment was intended to show that the removal of volatile acids from the culture, accomplished by the thorough washing with hydrogen gas, did not result in an alkaline reversion in the medium. The results are shown below. It will be seen that the preliminary expectations were only partially borne out. The plain broth used had been shown to contain sufficient native sugar to give the typical appearance of a Russell tube with typhoid and dysentery bacilli.

Comparison of the P_H value attained after incubation by the stoppered tubes with those of similar tubes incubated in the anaerobic jar

showed that the degree of acidity attained by the two sets were the same. Thus, the amount of oxygen contained in the stoppered tube had no measurable influence on the final reactions. That the oxygen in a closed system is quickly removed is strikingly shown by Wheeler [1] and by Novy.[7] The results shown in table 1 were of interest in that they show an apparent sharp division of the organisms tested into 2 groups: the typhoid, dysentery and paratyphoid A agreeing in their reactions, and the paratyphoid B, aertrycke and colon bacilli being distinctly different. In the former group, the initial P_H was lowered on incubation from 7.4 to 7. Washing out the volatile acids with hydrogen restored the original P_H value in each case. Further treatment with air resulted in an alkaline reversion of moderate degree, to about 7.8. The second group of strains tested showed almost the same initial degree of acidity.

TABLE 1

HYDROGEN ION CONCENTRATIONS

Organisms	Original	After Incubation	After Washing with H_2 10 Min.	After Washing with Air 10 Min.
B. typhosus	7.4	7.1	7.4	7.8+
B. Shigae	7.4	7.0	7.4	7.8
B. dys. Flexner	7.4	7.0	7.4	7.8+
B. paratyphoid A	7.4	7.0	7.4	7.8
B. paratyphoid B	7.4	7.1	8.0	8.4+
B. aertryche	7.4	7.1	8.0	8.4+
B. coli	7.4	7.1	8.0	8.4

but the hydrogen washing resulted in an alkaline change far beyond the original value of the medium, to 8 in all cases. Further treatment with air rapidly increased the alkalinity to 8.4 or more, the limits of the phenol red range being passed. It would appear, then, that the former group is capable of producing under anaerobic conditions a certain amount of acid which is volatile in hydrogen, indeed, that as far as this experiment goes, all the acid is readily carried off, leaving the medium at its original P_H value. The medium then contained, however, a substance which immediately developed alkaline properties on exposure to air. In the second group the experiment shows about the same degree of acid produced. On washing with hydrogen, however, and before treatment with air, the reaction becomes more alkaline than the original medium. This seems to show that these strains are capable of producing an alkaline substance under anaerobic conditions; and if this be admitted, they must also have produced more acid than did those of the former

[7] Jour. Infect. Dis., 1924, 36, p. 343.

group in order to have attained the same degree of acidity. They also produced the substance found in the former group which becomes alkaline on exposure to air, as is evidenced by the still greater alkalinity observed when air was passed through the tubes. In this group the presence of preformed alkali under anaerobic incubation prevents us from concluding that all of the acid produced is volatile, although there is nothing in the experiment to negative that supposition. It will be recalled that in exper. 1 and 2 the paratyphoid group strains showed no acidity of the slant with Andrade indicator and much less with phenol red than did the typhoid, dysentery, and colon strains. The only exception was the paratyphoid A strain which appeared to occupy an intermediate position. The acidity shown by colon strains in the earlier experiments is easily accounted for by the ability of this organism to ferment the lactose in the Russell tube and the consequently far greater amount of acid produced.

That the alkaline reaction in the butt of the Russell tube which develops after several days' incubation is not due to diffusion of alkaline substance from the slant, is shown by the fact that when such a tube is inoculated on the slant only, no such change occurs in the butt. The development of alkalinity comes with the gradual penetration of oxygen into the butt beyond the needs of the bacteria resident there.

It appears evident from the experiments described that all the members of the colon-typhoid group when grown anaerobically on Russell's medium produce a substance which, itself without inflence on the reaction of the medium, is changed into an alkaline reacting substance in the presence of oxygen. Exper. 7 would indicate that this change is perhaps reversible, and that on subsequent removal of oxygen the alkalinity also disappears.

The nature of this readily oxidizable substance and the materials from which it may be produced are matters of interest. When alkali is produced in a medium containing protein or its derivatives it has usually been assumed that the change was evidence of the production of ammonia or closely related amino products. Ayers and Rupp [8] have presented evidence in support of their belief that the alkalinity may be due to the transformation of readily oxidizable organic acid salts, such as sodium formate, into the corresponding bicarbonates. In buffered synthetic mediums containing an ample number of hydroxyl ions this is readily understood, but in a medium containing only small quantities of

[8] Ibid., 1918, 23, p. 188.

alkaline ions in such combination as to be attacked by the organic acids resulting from the primary fermentation, their explanation seems less tenable. For this reason we have set up the following experiments in order to ascertain the source from which the alkali-producing substance came.

Exper. 10.—A sugar-free plain agar medium was prepared by fermenting out the sugars present in the meat extract with a strain of Flexner dysentery bacilli. Tubes were filled with this medium in the same manner as with Russell's. They were colored with phenol red and adjusted to a P_H value of 7.4. They were then inoculated with representatives of the different groups under consideration. The results were noted after 24 hours' incubation anaerobically, and after 1 hour's exposure to the air (table 2).

TABLE 2

SUGAR-FREE AGAR

Organisms	After 24 Hours' Anaerobic Growth		After One Hour in Air, Slant	After 24 Hours in Air, Butt
	Slant	Butt		
Typhoid	No change	No change	Alkaline	Alkaline
Flexner dys.	No change	No change	Alkaline	Alkaline
Shiga dys.	No change	No change	Alkaline	Alkaline
Paratyphoid A	Very slight alkalinity	No change	Alkaline	Alkaline
Paratyphoid B	Slight alkalinity	No change	Alkaline	Alkaline
Gaertner	Slight alkalinity	No change	Alkaline	Alkaline
Aertrycke	Slight alkalinity	No chaneg	Alkaline	Alkaline
Colon	Slight alkalinity	No change	Alkaline	Alkaline

This experiment shows that the alkali production proceeds apparently uninfluenced by the absence of appreciable quantities of fermentable carbohydrate. The production of measurable quantities of alkali anaerobically by the members of the paratyphoid and colon groups, the intermediate position of the paratyphoid A, and the almost immediate production of an alkaline reaction in the slants by the remaining strains and in the butts of all when exposed to the air confirm the results of former experiments.

Exper. 11.—This was the same as the preceding experiment, except that 0.1% of dextrose was added to the sugar-free agar. In this medium all the strains reacted in the same way. Slant and butt were both acid after anaerobic incubation (gas was, of course, formed by the paratyphoids and colon), while on exposure to air the slants all became alkaline, the butts remaining acid. The alkaline reversion was distinctly slower than in the former experiment, and it could not be said that there was in this experiment a greater degree of alkaline change than in exper. 10.

It appears that the alkali, both that formed anaerobically by the paratyphoids and colon and that appearing only on exposure to air, is formed independently of the presence of acid fermentation, or indeed of the presence of fermentable carbohydrate. It must, then, be the result of the metabolism of the extractives or of the peptone. Experiments similarly planned to determine which of these constituents of the medium was mainly concerned proved inconclusive, as some alkali was produced with either in the absence of the other. It appeared, however, that the reaction was more intense in the peptone medium than when the extract only was employed. The element of growth activity entered into this comparison, however, sufficiently to make any conclusions unsatisfactory.

SUMMARY

Most, if not all, of the acid produced by the fermentation of carbohydrate by the organisms studied is volatile; that is, it may be removed from a liquid medium by washing with hydrogen. The question as to how much of this acid is carbonic and how much formic or other volatile organic acid which might easily be oxidized to carbonic acid, we leave unanswered. It appears evident, however, that these volatile acids do not diffuse out of the agar medium sufficiently to change its reaction from the acid side. This is shown by the results of anaerobic cultivation when all the conditions are made favorable to the complete diffusion of any volatile acid. We have no confirmation of the point made by Wheeler that the production of volatile acid is favored by the presence of oxygen. It would appear from our work that they are easily produced in its absence.

The alkaline fermentation shown by the colon typhoid group is shown to be of two types. One type common to all of the organisms studied is manifested by the production of an alkaline reaction only in the presence of oxygen. We believe that we have shown conclusively, however, that this alkaline substance is formed in two stages: first, the formation of a mother substance as a result of the activities of the growing organism; and second, the oxidation of this substance by air and independently of further bacterial growth into the final product which gives the alkaline reaction. The second type of alkaline fermentation, shown only by the members of the paratyphoid and colon groups, is characterized by the production of an alkaline substance in the absence of oxygen. As far as our observations go, it would appear that both

these types depend on the presence of nitrogenous bodies rather than on the refermentation of the products of acid change as suggested by Ayers and Rupp.

CONCLUSIONS

While the acids produced in the fermentation of dextrose in the Russell double sugar medium are mainly, if not entirely, volatile, their diffusion out of the medium is not the cause of the alkaline reaction shown on the slant when typhoid and related organisms are grown.

This reaction is due to excess of alkaline substance produced from nitrogenous elements in the presence of oxygen.

In the absence of oxygen a "mother substance" is formed, which rapidly becomes alkaline when exposed to oxygen.

The ultimate alkaline reversion in the butts of Russell tubes is the result of the diffusion into the butts of oxygen rather than of the alkali formed in the slant portion of the tube.

The paratyphoid and colon groups appear capable of producing an alkaline reacting substance under anaerobic conditions, thus evidencing a definite difference in metabolism which may prove worthy of further study.

No suggestion is made as to the chemical nature of the substance which is so easily oxidized into an alkaline substance.

OBSERVATIONS ON 6,500 CASES OF LOBAR PNEUMONIA AT THE COOK COUNTY HOSPITAL, CHICAGO

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The importance of the rôle played by any disease can be determined only by making observations from time to time on its incidence and mortality. It is especially desirable that such data concerning lobar pneumonia be ascertained at this time because of the activity in search of a specific treatment for this disease. The true case fatality is unknown. A near approach to it for males in the decennium 21 to 30 years is 9.9%. This was the rate in 2,953 cases in the United States Army Camps from September, 1917, to March 1918. Practically all of the men in these camps were in this age group, and, being selected, were relatively free from physical defect.

Much of the data that has been published refers only to hospital patients. It is recognized that these include an increased proportion of the more serious cases. It is reasonable to believe that many cases of lobar pneumonia in the general community never get into records. Some are undiagnosed, others are not reported, and a third group are not specially defined. This last number is decreasing. In 1910, the deaths from undefined pneumonia in the registration area of the United States equaled the deaths due to lobar pneumonia. Requests for greater accuracy in reports resulted by 1920 in a decrease of the figures for undefined pneumonia to one-eighth of the value of 1910, and doubled those for lobar pneumonia. The change involved particularly the 20-39 year age group.

In order to have significant findings it is necessary to have statistical figures involving groups sufficiently large that any chance variation in the original data will not vitiate the interpretation of the results. Figures below 100 have been shown to be unreliable. From 100 to 1,000 there is a rapid increase in their value. And from that figure upward they permit deductions which are increasingly dependable. The following observations are based on 6,531 case histories from Jan. 1, 1917. to July 1, 1924, filed as lobar pneumonia at the Cook County Hospital.

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This institution some years cares for more than 1,200 cases of lobar pneumonia, and presents an unusual opportunity for observing the disease.

GENERAL CASE FATALITY

The case fatality in this entire group was 36%. This includes all deaths in which lobar pneumonia was considered the cause of death, regardless of any associated circumstances.

TABLE 1

General Mortality of Lobar Pneumonia

Location	Total No. of Cases	Case Fatality, %	Period and No. of Years Included	Average Case Incidence per Year
Charity Hospital New Orleans (Osler, in Townsend and Coolidge)	3,969	38.01	1830-79 49	79
Montreal General Hospital (Osler, in Townsend and Coolidge)	1,012	20.4	1853-87 34	29
Massachusetts General Hospital (Townsend and Coolidge)	1,000	25.0	1882-87 65	15
Germany (Fränkel and Reiche [1a])	1,130	19.3	1894	?
Magdeburg, Germany (Aufrecht, quoted by Anders)	1,501	16.8	1880-96 16	95
Mt. Sinai Hospital, New York (Meyer)	500	18.88	1888-98 10	50
Boston City Hospital (Sears and Larabee)	949	30.6	1895-1900 5	185
Pennsylvania Hospital (Norris)	500	27.6	1897-1901 4	125
Montreal General Hospital (McCrae, Fysche and Ainsley [1b])	486	21.2	1895-1903 8	61
Breslau Medical Clinic (Baeck)	304	16.7	1892-1903 11	27
Philadelphia General Hospital (Ashton and Landis)	991	54.3	1897-1901 4	247
Johns Hopkins Hospital (Chattard)	685	30.39	1889-1905 16	42
Massachusetts General Hospital (Shattuck and Lawrence)	3,291	27.1	1889-1917 28	117
Nine hospitals in New York City [1c]	1,462	42.8	1920-21 2	731
Bellevue Hospital (New York City report)	1,381	24.4	1921 1	1,381
Total	19,161	28.1 weighted average		
Cook County Hospital (Tice and Herndon)	3,439	37.1	1911-16 5½	625
Cook County Hospital (present report)	6,531	36.0	1917-23 7½	870

The most extensive figures at hand on the case fatality of lobar pneumonia are those of Wells,[1a] who in his accumulated data of 100 years statistics includes 359,797 cases, with a case fatality of 21.8%. A comparison of the present series with others is made in table 1. Two facts stand out: first, the wide range in the case fatalities, from

[1a] Ztschr. f. klin. Med., 1894, 25, p. 230.
[1b] Am. Med., 1904, 7, p. 135.
[1c] New York City, Report of Dept. of Public Welfare, 1921.
[1d] Jour. Am. Med. Assn., 1902, 39, p. 1231.

16.7 to 54.3%; and second, the long period of years with few cases in any year in many reports. Eight of the summaries have an average of less than 100 cases a year, whereas in the present series the average is 870 cases.

RECENT AS COMPARED WITH PAST CASE FATALITIES

The different periods covered by the individual investigations included in table 1 present an opportunity to compare the present case fatality of lobar pneumonia with that of previous years. The results up to 1879 at New Orleans show a slightly greater case fatality than recently at the Cook County Hospital. From 1888-1898 at the Mt. Sinai Hospital in New York the case fatality was half of what it

TABLE 2

Time Distribution of Cases of Lobar Pneumonia at the Cook County Hospital

Year	Total Cases	Case Fatality in Percentage
1901 (Ingals)	338	36.0
1911 (Tice and Herndon)	918	38.8
1912 (Tice and Herndon)	493	37.1
1913 (Tice and Herndon)	600	34.6
1914 (Tice and Herndon)	597	38.6
1915 (Tice and Herndon)	331	29.7
1016 (Tice and Herndon)	500	40.2
1917	1,395	37.6
1918	978	41.4
1919	354	35.8
1920	524	38.7
1921	488	26.6
1922	776	37.2
1923	850	35.8

averaged during the past few years at the Cook County Hospital. The Philadelphia General Hospital had a much higher case fatality from 1897-1891 than that of the present series. But the Montreal General Hospital and Massachusetts General Hospital in the years preceding 1887 had a much lower figure.

Table 2 shows the case fatalities at the Cook County Hospital at different periods. The highest case fatality occurred in 1918, 41.4% succumbing; the lowest was in 1921 when only 26.6% died. The latter figure is comparable with the 1921 rate of 24.4% at the Bellevue Hospital,[2] a similar type of hospital (table 1). During the other years covered in this series, the case fatality varied slightly, ranging from 35.8 to 38.7%. The increased rate in 1918 was present throughout the year. The individual months, except May, June and August, had higher

[2] New York City, Report of Bellevue and Allied Hospitals, 1921, p. 73.

case fatalities than the weighted averages for the corresponding months for the entire 7½ years. If the influenzal epidemic did not occur until September, it had no opportunity to influence the mortality of the first three-fourths of the year. Many believe, however, that influenza was present in the spring. In 1921, the low rate was also uniform, although June, July and September had higher case fatalities than the weighted averages for the corresponding months throughout the entire period.

This striking difference in the case fatalities of 1921 reflected a general decrease in the incidence and death rate of lobar pneumonia in that year. Only 488 patients were treated for the disease in the Cook County Hospital during the year, the smallest number in any one year during the period observed, and but two-fifths of the total for 1917. In the City of Chicago, according to a personal communication from the Health Commissioner, there were but 1,087 deaths from lobar pneumonia in 1921, whereas in 1920 there were 2,123, and in 1922, 1,508 deaths. In the entire registration area of the United States there were but 37,871 deaths from lobar pneumonia in 1921, while in 1920 there were 64,001, and in 1922, 47,031.

Fortunately, there is an opportunity of comparing the present case fatality at the Cook County Hospital with the past. In 1902, Ingalls [3] made an analysis of 338 cases occuring from Jan. 1, 1901, to April 1, 1902. He found a case fatality of 36%. In 1916, Tice and Herndon reported for the period of Dec. 1, 1910, to May 1, 1916. Their data included 3,439 cases, ranging from 331 to 918 per year, with case fatalities varying from 29.7 to 40.2%. Their case fatality for the entire period was 37.1%.

One of the longest periods over which the case fatality of lobar pneumonia has been observed is 95 years. These patients were at the Massachusetts General Hospital. The results for the years 1822-1879 were reported by Townsend and Coolidge.[4] Shattuck and Lawrence [5] brought the series down to date. They found variations in the annual case fatality of from 16 to 40%. When they grouped the cases by decades, the case fatalities for the 10 year periods were between 25 and 31%. They claimed that the death rate by decades is showing no rise, that the case fatality has made no permanent change and none is to be expected as the result of treatment until a specific is discovered for lobar pneumonia.

[3] Jour. Am. Med. Assn., 1902, 39, p. 1301.
[4] Tr. Am. Climat. Assn., 1889, 6, p. 22.
[5] Boston Med. & Surg. Jour., 1918, 178, p. 245.

RELATION OF SEX TO CASE INCIDENCE AND CASE FATALITY

Table 3 gives the distribution according to sex of the cases of lobar pneumonia at the Cook County Hospital and in other series. Four-fifths of the patients in the present series were males, agreeing with the common assertion that males are more frequently affected than females. The usual explanation of this belief is that men are more frequently exposed to such predisposing factors as cold, changes of weather, dampness and trauma. The case fatality has been just a trifle higher for the men, with the variations in the case fatality by years being practically identical for both sexes. All of the other reports show a marked preponderance of males over females. As at the Cook County Hospital, the observed case fatality has frequently been practically the

TABLE 3

Distribution of Cases Elsewhere According to Sex

Investigator	Incidence		Case Fatality in Percentage	
	Male	Female	Male	Female
Townsend-Coolidge	724	276	25	25
Fränkel-Reiche	979	239	18.3	23
Aufrecht	1,223	278	16.5	18.0
Meyer	348	152	16.6	23.6
Sears	714	235	36.5	34.0
Norris	382	118	30.0	20.0
Baeck	255	90		
Chattard	533	125	28.8	36.8
Shattuck	2,052	976	30.7	26.8
Present series	5,325	1,206	36.5	33.0

same for the two sexes. Four of the writers noted a higher case fatality among the females, four a higher for males, and one reported the same figure for both sexes.

SEASON AND LOBAR PNEUMONIA

Separating the Cook County Hospital cases according to their month of entrance and summarizing the figures for the years covered, shows August as the month of lowest number of hospital entrances with lobar pneumonia. From then until March there is a steady rise, with the greatest and sharpest increases in December and January. In April and May there is a steady, rapid decrease; with a sudden, almost precipitous drop in June; then a continued, gradual decline through August. Sixty-two % of all the patients entered in January, February, March and April.

Comparing these observation with those in other reports, Aufrecht, as quoted by Anders, Sears and Larabee,[6] Ashton and Landis,[7] Chattard,[8] and Shattuck and Lawrence, all found the greatest incidence in March. Baeck,[9] Townsend and Coolidge, and Norris[10] found April had more cases. Ziemissen, as quoted by Baeck, claimed the maximum frequency to be in the spring months. Siebert[11] reported February and March had the greatest incidence. According to Anders,[12] Harrington noted that March, April, May and June led in London, and Hirsch stated that March, April and May had the most cases and June, July and August the fewest. August was the lightest month in 6 reports, July in 2 and September in 1.

The highest average case fatality for the 7½ years in the present series was in October, 41.8%; the lowest in June, 32.4%. For the entire period the average case fatality was 35% or less in March, May, June, July and December. The other 6 months had case fatalities between 35 and 40%. These are not large differences, but when the case fatalities for the months of individual years are examined, it is found that with but an occasional variation there is a wavelike constancy of fluctuation in the case fatality, with crests in October, January and April of each year. It must be borne in mind that these case fatalities refer to the cases entering the hospital in the months designated, though the death occurred in a subsequent month.

THE INFLUENCE OF AGE ON LOBAR PNEUMONIA

"Up to the sixth year the predisposition to pneumonia is marked; it diminishes to the fifteenth year, but then for each subsequent decade it increases" (Osler). In the present series there is a greater incidence for the first two years of life than for the following three, 214 to 180; greater for the first five years of life than for the 5 to 9 year age group, 394 to 252, greater for the first decennium than for the second, 646 to 495; then there is a sharp rise to 1,336 for the third decennium and an even further advance to 1,488 for the fourth, the peak of the incidence curve. Receding slightly there is an incidence of 1,265 cases in the fifth decennium, then a marked drop to 739 for the sixth and an even smaller figure, 502 cases, for the "60 years and over" group.

[6] Med. & Surg. Rep. Boston City Hosp., 1901, 12, p. 1.
[7] Am. Jour. Med. Sc., 1905, N. S., 129, p. 952.
[8] Maryland Med. Jour., 1906, 49, pp. 184, 400, 433.
[9] Inaugural Dissertation, Breslau, 1904.
[10] Am. Jour. Med. Sc., 1901, 121, p. 684.
[11] Am. Jour. Med. Sc., 1882, N. S. 83, p. 108.
[12] Am. Med., 1904, 8, p. 407.

The case fatality for the different age groups is essentially a curve beginning moderately high in the first 10 years of life at 20.3%, going down to 13.5% in the second decennium, then rising steadily with each succeeding decennium in order.

With two minor exceptions, the case fatalities in the age groups at other hospitals correspond to those at the Cook County Hospital. Meyer [13] had a lower case fatality in the 20-29 year group than in the second decennium. Chattard reported the first decennium with a lower case fatality than the second. Shattuck and Lawrence, commenting on the high case fatality in the patients over 60 years of age, expressed the belief that arteriosclerosis is the factor causing the increase.

TABLE 4

Total Result for White and Colored Races for Entire Period at Cook County Hospital

Year	White				Colored			
	Male		Female		Male		Female	
	Total Cases	Case Fatality	Total Cases	Case Fatality	Total Cases	Case Fatality	Total Cases	Case Fatality
1917	903	39.7	171	36.2	284	33.3	37	27.0
1918	580	44.8	135	41.4	209	31.1	54	44.4
1919	195	38.4	51	37.2	91	31.8	17	23.5
1920	310	40.3	78	41.0	110	32.7	26	38.4
1921	300	30.3	70	20.0	85	24.7	33	12.1
1922	477	38.7	85	36.4	170	33.5	44	36.3
1923	590	40.5	133	33.7	376	28.4	109	31.1
1924	394	37.3	95	28.4	251	27.8	68	32.3
1917–1924	3,749	39.2	818	34.7	1,576	30.6	388	31.9

LOBAR PNEUMONIA IN THE NEGRO RACE

In table 4 the case fatalities for the white and negro races are given. Every year the case fatality in the male negroes has been from 5 to 13% below that of the male whites, with a difference of 8.8% for the entire 7½ years. The female negro case fatality has been 4.6% less than that of the white females in the same period. In 2 years, however, the difference favored the white females.

When racial groups are further subdivided according to age, there is seen a greater case incidence among the whites in the older age groups in which the mortality is higher. For example, the incidence peak is in the 40-49 year group for the white males and in the 20-29 year group for the colored males. When the age standardized fatality rates are calculated, using the age groups of the white males of 1917 as a standard,

[13] Mt. Sinai Hospital Rep., 1898, 1, p. 29.

the case fatality for all persons is 37.1%; white males, 37.6%; colored males, 34.9%; white females, 35.8%; colored females, 35.9%. The lower case fatality among the negro males persists through this age standardization. It is due to the relatively lower rates in 3 upper age groups of 40-49 years, 50-59 years, and 60 years and over, in which differences of 5.7, 12.3 and 14.7%, respectively, favor the colored males. Had these groups in the colored males as high a case fatality as in the white males, the expected case fatality of the colored males, with the above age standardization, would have been 38.6%.

Information concerning the incidence and case fatality of lobar pneumonia among negroes is scanty. Chattard [11] found a case fatality of 31.2% in 250 cases, compared with 30.6% in the native born white. Osler [14] stated that the disease is more fatal among the negroes, citing the Charity Hospital of New Orleans as his source of information and qualifying his remark by saying the difference was less marked at the Johns Hopkins Hospital. Miller and Lusk [15] found the negro troops at Camp Dodge more susceptible to lobar pneumonia. In their series, there were 116 negroes and 115 whites, although the colored troops in camp were only one-sixth the number of whites. The case fatality of the negroes was 12.9%; that of the whites, 9%.

WARD MORTALITIES

In the Cook County Hospital there are 4 male and 2 female medical wards. Two of the former are double wards, caring for twice the number of patients as the single wards. All the patients in all of these wards are over 12 years of age. The children are placed in a separate building. Scattered throughout the hospital there are occasional cases of lobar pneumonia, so located mistakenly with a wrong diagnosis, or purposely because of some complication requiring special treatment. The case fatality of the latter is high, 58.4%, as these cases include those with complications of surgical nature, traumas, contagious diseases, tuberculosis, etc.

The lowest average case fatality for the 7½ years in a male ward was 36%. In 5 of the years this ward had a higher case fatality than at least one of the other male wards. The highest average case fatality for the entire period in a male ward was 39.7%. In 4 years this ward had a case fatality lower than at least one of the other male wards. One of the female wards had an average case fatality of 34.5%, the

[14] The Principles and Practice of Medicine, 1917, p. 74.
[15] Jour. Am. Med. Assn., 1918, 71, p. 702.

other 37.8%. In 3 of the years the former ward had a higher case fatality than the latter. No ward had a consistently higher or lower case fatality than any other ward.

Since the case fatality in the age groups over 40 years is so much greater than below that age, it is advisable to observe if this was a factor responsible where marked differences existed in the case fatalities in the various wards. In 1918, one ward had a case fatality of 48.3%, another 39.5%. In 1919, one had a case fatality of 41.1%, another 25%. In 1922, one had a case fatality of 45.5, another 32.6%. In 1923, one had a case fatality of 42.9, another 31.5%. When the age standardized case fatality rates are calculated for these wards during the designated years, these differences are seen to be real and not apparent. In the same order the results are, 1918, 50.5 and 40.5%; 1919, 43.2 and 21.8%; 1922, 41.3 and 36.8%; 1923, 43.7 and 32.5%.

DISCUSSION

No attempt is made in this report to eliminate any special type of case. Many of the deaths occurred before sufficient time had elapsed for the initiation of hospital care. When the data were investigated for the months of January, February and March, 1923, it was found that 50% of the deaths took place within 48 hours after the patient entered the hospital. In institutions having a large yearly entry of a similar type of patients comparably high death rates are found, e. g., in the Philadelphia General Hospital, Boston City Hospital and Bellevue Hospital. Whatever the rôle of predisposing factors—alcoholism, undernourishment, lack of earlier medical attention—the fact is that the patients have lobar pneumonia and contribute to its incidence and mortality.

The variations in the case fatality in different years, 26.6 to 41.4% in the present series, illustrate the differences in virulence of infection that occur from year to year and that form part of the basis for the conflicting opinions regarding the trend of the disease and its treatment. These yearly variations are leveled when the years are considered by groups. Thus Shattuck and Lawrence, finding case fatalities between 16 and 40% in different years, showed that by decades the gap narrowed to percentages between 25 and 31%. And Tice and Herndon,[16] at the Cook County Hospital found the case fatality varied from 29.7 to 40.2% in single years, which data, when summarized show a case

[16] Ill. Med. Jour., 1916, 30, p. 388.

fatality of 37.1% for the entire 5½ years observed as compared with 36% for the 7½ years in the present report.

Although a marked difference in the hospital incidence in the two sexes is shown, the case fatalities were practically the same. Other series show a similar phenomenon. The elements of exposure, cold, trauma, etc., as predisposing factors and obviously more important with the men, suggest an adequate explanation for the greater number of cases among the males.

One of the most interesting facts brought out in the present investigation is the case fatality of lobar pneumonia among the negroes. It has always been claimed that they have less resistance than the white race to the disease. Few figures are cited, usually only opinions being given. There is rarely an opportunity of making comparisons under identical conditions of hospitalization. Fortunately, this could be done in the present report. The case fatality for the two races was the same up to the "40 years and over" groups, above which the negroes had the greater resistance. The number of patients is sufficiently large, and the results so consistent through the passing years that they minimize the probability of their being chance findings. Various suggestions may be offered to explain these facts. For example, it may be claimed that the recent migration of the negro northward has brought a larger percentage of the strong, hardy members of the race with a naturally greater resistance than general. However, most of these newcomers are under 40 years of age, in which class the case fatality has been the same as in the white race. Also, an unselected group of 170 of the negro patients with lobar pneumonia, all of them from 20 to 40 years of age, entering the hospital between Jan. 1, 1923, and June 1, 1923, were checked up, and 65% had been in Chicago more than two years, a great many from 10 to 20 years. It is likely that among the "40 years and older" group a far greater percentage had spent many years here. Their veracity, unintentionally or otherwise, concerning their age may be questioned, and age in an adult negro is not easily guessed. Granting their statements as correct, another contention may be that only the most fit and hardy survived childhood, constituting an unusually vigorous class because of the elimination of the weaker members in earlier life.

The resistance of the race to lobar pneumonia in Chicago cannot be determined without consideration of the case incidence of the disease. This phase of the question can be approached here indirectly. Negroes made up 4.1% of the population of Chicago in 1920. They accounted

for 8.4% of the deaths from lobar pneumonia in the entire city of Chicago in the period 1917-1924. Assuming from the results at the Cook County Hospital that the case fatality in the entire city in this infection was no greater in the negroes than in the whites, the case incidence in the negro race was approximately twice that in the white race.

Incidentally, negroes constituted 30% of the total number of patients with lobar pneumonia at the Cook County Hospital, seven times their ratio in population in the city—mute testimony of their relative indigence.

The marked increase in the incidence of lobar pneumonia in the late winter and early spring months is readily perceived. It is a common finding in reports of large series of cases. Among the theories advanced in its explanation is that of Siebert that the "rise of the relative humidity of the atmosphere during descending or existing low temperatures is attended by an increased number of cases of pneumonia. The sudden appearance and long prevalence of a cold, moist atmosphere, especially influences the origin of lobar pneumonia." Anders claims "The major influence exerted by seasons probably is not direct, e. g., by a lowering of the bodily resistance due to a low temperature, a high barometric pressure, direction and velocity of winds, etc., but indirect, namely, by bringing about that effective element in the causations, concentration, and increased virulence of the specific person in consequence of closed doors and windows, and lack of ventilation."

The incidence and case fatality in the various age groups give us the most direct evidence regarding the seriousness of the disease. The effect of age on the prognosis is markedly emphasized therein. The very young and very old have little chance of survival. The youth has everything is his favor.

It is commonly considered that pneumonia of the lobar type is a disease of early adult life. If this be true, there should be a greater case incidence in the age groups of this period. 43.2% of all the cases of lobar pneumonia in the hospital occurred in the 20-29 and 30-39 year age groups; 37.5% of the city's population is in these decenniums; 34% of the deaths from lobar pneumonia in the hospital, and 32.4% in the entire city during the years 1917-1924 were in these age groups. These percentages show that the hospital incidence in the 20-39 year age group is slightly more than proportional to the population. By a similar process of determination, the incidence in the "under 10 years" and "10-19 years" age groups was less, and in the "40 years and older"

group greater than the proportion of population. The case incidence in the hospital, directly, and the age distribution of the deaths from lobar pneumonia in the entire city, indirectly, tend to show this infection to be an important disease of all ages, but particularly of later adult life.

The ward case fatalities permit reflection on the citation of uncontrolled statistics in support of any form of treatment of lobar pneumonia. Frequently, the number of cases included is small, and more than one hospital may be included. As shown in table 2, there is normally a marked variation in the case fatalities in different years. Comparison with other hospitals in the same year requires consideration of many local factors. But in these figures for the several wards, comparison is possible with exactly the same kind of cases, under like conditions, at one time. Even here there are differences in the case fatality in the same year. The efficiacy of any plan of treatment should be measured by its ability to produce consistently a greater decrease in case fatality than occurred in similar wards as shown here.

SUMMARY

In the seven and one-half years from Jan. 1, 1917, to July 1, 1924, there were 6,531 cases of lobar pneumonia at the Cook County Hospital, Chicago, with a case fatality of 36%.

The case fatality in the individual years varied between 34 and 41.4%, with the exception of 1921 when only 26.6% died. The decrease in 1921 occurred in both sexes, in negroes and in whites, and in all age groups. The death rate from this infection was markedly lower in the entire country in that year. There was no significant difference in the case fatality for the entire period in the two sexes.

The case fatality among the negroes was from 5 to 13% below that of the whites in the different years. This was due to a lower case fatality in the negroes in the "40 years and over" group. The case incidence among the negroes in Chicago during this period was apparently twice that of the whites.

The age incidence in the 6,531 cases showed a large number in the "under 5 years" group, the fewest in the second decennium, with a steady increase through the "40 to 49 year" group, followed by a decline so rapid that the number in the "60 year and over" group was very small.

The case fatality was lowest in the "5-9 year" group, and from that point increased proportionately with the age.

The greatest number of persons with lobar pneumonia entering the hospital during the year was in the months of January through April. The lowest number was in August. The greatest case fatality was in October, the lowest in June, the variation throughout the composite year being from 32.4 to 41.8%.

There was only 5% difference in the total number of case fatalities of the separate wards for the entire period. Differences in individual years were not accounted for on a basis of age.

THE VIABILITY OF B. COLI AND B. AEROGENES IN SOIL

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Very large numbers of Bacterium coli are introduced into the soil in the process of manuring. Little is known as to the viability of these bacteria in soils. We know something of their viability in water, and Murray [1] has shown that in manure piles they are rapidly crowded out by other organisms. If the soil harbors B. coli for long periods of time, it is evident that bacteria in soils will be found in surface water draining from those soils. If, on the other hand, B. coli rapidly disappears from soils, the presence of this organism in water is a better index of human fecal pollution.

It is now well established that the so-called colon bacilli which are found in the feces of warm blooded animals and most of those found in soil differ in certain cultural characteristics.[2] The fecal "colon bacilli" are largely Bacterium coli, the methyl red positive and Voges-Proskauer negative organism, while in the soil most of the organisms of this group are found to be Bacterium aerogenes, an organism showing opposite methyl red and Voges-Proskauer characters. Possibly neither of these organisms is a single species, but, following Levine,[2] they will be grouped as single species. According to Koser,[3] the relatively few B. coli that are found in soil differ from the fecal strains in that they possess the ability of using citrates as a source of energy.

That B. aerogenes in soil may in some cases be the "B. coli" found in routine water analysis has been known for some time. B. aerogenes is present in most soils,[2] but not in very large numbers. Their function there is known, although Pillai [4] has demonstrated significant nitrogen fixation by Bacterium pneumoniae, which he considers identical or closely allied to B. aerogenes. B. coli did not fix nitrogen.

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[1] Virginia Agric. Exper. Sta., 1917, Technical Bull. 15.
[2] Iowa State College, Eng. Exper. Sta., 1921, Bull. 62.
[3] Jour. Bacteriol., 1924, 9, p. 59.
[4] Dissertation, Leipzig, 1908.

Young and Greenfield [5] have shown that B. coli survived in soils for four years. In this work it was shown that the moisture was a factor in the viability of B. coli in soils.

Savage [6] has shown in a study of "made-soils," which consisted of ordinary dust bin refuse, street sweepings and street scrapings, that B. coli gradually diminished and disappeared. They were gone after 4 years. In this work there was no differentiation made between B. coli and B. aerogenes.

In order to trace the disappearance of B. coli and B. aerogenes from feces and pure cultures when introduced in soil, the following experiments were performed: Twelve samples of soil were tested in order to find one free from B. aerogenes, showing its rather general distribution in soil. A sassafras loam was finally chosen. It was air dried and passed through a 2 mm. sieve and placed in 200 gm. amounts in 4 glass tumblers. Into 2 of these was thoroughly mixed 1 gm. of fresh cow feces. The other 2 were kept as controls. Thirty c c. of water was added to each tumbler to bring to 50% saturation, and fresh water was added frequently to keep the soil moist. (The saturation point of sassafras loam is 30% moisture. There was actually therefore 15% moisture in this experiment).

Neither B. coli nor B. aerogenes was present in the soil, and B. aerogenes was not found in the cow feces.

At intervals, the approximate number of B. coli was determined as follows:

Dilutions were made into sterile water blanks. Five tubes of lactose gentian violet broth [7] in fermentation tubes were inoculated with 1 c c. of each of the dilutions. After from 24 to 48 hours' incubation tubes showing gas were streaked on triple dye agar [8] to confirm the presence of B. coli and to differentiate from B. aerogenes. The positive plates were recorded and the number of B. coli was estimated from the tables of McCrady.[9] He gives two sets of figures in table 1 for estimating the number of B. coli per 100 c c. of water; positives with 10 c c. and 1 c c. and positives with 10 c c., 1 c c. and 0.1 c c. The dilutions are in multiples of 10 as was the case with our dilutions. For our computations, table 1 was used directly. For example, when we used dilutions of 1:1,000,000, 1:100,000 and 1:10,000, readings were made directly from the column, positives with 10 c c., 1 c c. and 0.1 c c. The number obtained was multiplied by 100,000 and then divided by 100; or, as was done in actual practice, the number found was simply multiplied by 1,000 to obtain the number of B. coli or B. aerogenes per gram of soil.

[5] Am. Jour. Pub. Health, 1923, 13, p. 270.
[6] Jour. of Sanitary Inst., 1903-4, 24, p. 442.
[7] Hall and Ellefson: Jour. Bacteriol., 1918, 3, p. 329.
[8] Jour. Infect. Dis., 1924, 34, p. 585.
[9] Pub. Health Jour., 1918, 9, p. 201.

These tables are calculations from the formula for the most probable number of colon bacilli from a series of fermentation tubes. The formula is as follows.[9]

$$(p + q) \log 0.9 + (r + s) \log 0.99 + (t + u) \log 0.999 = \frac{p \log 0.9}{1 - 0.9^x} + \frac{r \log 0.99}{1 - 0.99^x} + \frac{t \log 0.999}{1 - 0.999^x}$$

in which $(p + q)$ = no. of tubes inoculated with 10 c c. sample
$(r + s)$ = no. of tubes inoculated with 1 c c. sample
$(t + u)$ = no. of tubes inoculated with 0.1 c c. sample
x = no. of fermenting organisms in 100 c c. sample. p, r, and t = no. of tubes giving positive results.

Occasionally, the soil from the control tumbler was also inoculated into broth, but neither B. coli nor B. aerogenes was ever encountered. the results from the two tumblers containing manure are given in table 1.

TABLE 1

THE DISAPPEARANCE OF B. COLI FROM SOIL TO WHICH 0.1% MANURE HAS BEEN ADDED

Days of Incubation	Number of B. coli	
	A	B
0	600,000	600,000
4	250 000	900,000
7	130,000	600,000
10	35,000	95,000
17	11,000	8 000
24	8,000	8,000
38	5,000	8,000
52	1,300	1,700
66	800	500
80	250	130
94	80	50
108	35	5
122	5	0
136	0	0
157	0	0

A and B are duplicate tumblers.

It will be noted that there was a gradual decrease in the number of B. coli in the soil, until they entirely disappeared. It would seem that the manuring of land may offer for a time a possible source of some of the B. coli found in routine water analysis.

In order to trace the course of disappearance of both B. coli and B. aerogenes in soil, another experiment was run. Here a Penn Loam free from colon bacilli was used, and 60 c c. of water per 200 gm. of air dry soil was added to bring the soil to 50% saturation. (The water holding capacity of Penn Loam soil is high. The saturation point is 60% water, so that the actual moisture in this experiment was 30%.) Instead of manure, 5 c c. of a 24-hour broth culture of B. coli and B. aerogenes was used for inoculation. Both strains were freshly isolated

from sewage and were probably of fecal origin. A mixture of cultures was also used for a third tumbler. The procedure of making dilutions and counting was as before. In the case of the soil containing both organisms a modification had to be made, since the B. aerogenes organisms very soon outnumbered the B. coli, and overgrew them on the plates streaked from dilutions. It was found, following the Eijkman [10] method, that the strain of B. coli used grew well at 46 C., but B. aerogenes did not develop. That all strains of B. coli will grow at 46 C. and all strains of B. aerogenes will not grow is not known, but offers an interesting possibility as a method of separation of the two species. From the tumbler containing both organisms, two sets of fermentation tubes were made. One set contained gentian violet and was incubated at 37 C. In this B. aerogenes developed very well. In the other no gentian violet was used and was incubated at 46 C. In this only B. coli developed.

The results are shown in table 2. The calculations were made from the McCrady tables as before.

TABLE 2

THE DISAPPEARANCE OF B. COLI AND B. AEROGENES FROM SOIL WHEN INTRODUCED IN PURE AND IN MIXED CULTURES

Days of Incubation	Pure Culture		Mixed Culture	
	B. coli	B. aerogenes	B. coli	B. aerogenes
0	9,000,000	13,000,000	1,300,000	800 000
4	2,500,000	8,000,000	170,000	1,300,000
11	800,000	2,500,000	17,000	.....
25	600,000	2,500,000	50,000	900,000
39	.....	.....	50,000	900,000
43	350,000	1,300,000	5,000	600,000
57	50,000	900,000	5,000	350,000
78	35,000	17,000	1,300	350,000
92	25,000	25,000	1,300	13 000
106	6,000	25,000	500	8,000
120	500	17,000	500	5,000
134	130	11,000	80	800
148	20	2,500	20	500
162	13	800	11	350
176	5	130	5	170
197	0	50	0	50
218	0	50	0	80
248	..	0	..	0

It will be noted that both B. coli and B. aerogenes diminished in numbers rapidly. These results confirm the results with the manure. Furthermore, they indicate that the strain of B. aerogenes used finds a habitat in the soil not much more suited for its development than B. coli. Since this strain was of sewage origin, it cannot be concluded that

[10] Centralbl. f. Bakteriol., I, O., 1904, 37, p. 742.

B. aerogenes is not endemic in the soil. The fact that 12 soils were tested to find 2 free from this organism would argue against this conclusion.

SUMMARY

The number of B. coli when added to a soil from feces or pure culture diminished until they disappeared.

The number of B. aerogenes when added to a soil in pure culture or mixed with B. coli diminished almost as rapidly as did B. coli.

B. aerogenes is found in many soils under natural conditions.

THE EFFECT OF CLOSTRIDIUM HISTOLYTICUM ON THE TYROSINE IN PROTEINS

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Weinberg and Seguin [1] in 1915 reported the isolation of Clostridium histolyticum (also called Bacillus histolyticus) from cases of gas gangrene in war wounds of French soldiers. It was described by them in 1916 [2] and since then has been isolated many times. The French investigators considered it a type of soil organism, and recently it has been isolated from the soil in California.[3] The name is derived from its ability to liquify living tissue with extreme rapidity. Its marked digestive action in the tissues surrounding wounds, especially those produced by projectiles, was observed by Legros and Vaucher,[4] and Wolf and Harris.[5]

Similar observations have been made by Nicholas,[4] and by Blanc and Pozerski [6] in horses and their experiments on guinea-pigs showed the same result.

There is a difference of opinion concerning the characteristics of the organism, because so many strains are known, and the work has been done under varying conditions.[7]

CHEMICAL CHANGES PRODUCED BY THE ORGANISM

Most of the work with the organism has been bacteriologic rather than chemical, with the result that little is known concerning the changes produced in the mediums in which it has grown.

Weinberg and Seguin [1] were unable to discover gas formation in any of their mediums and believed there was little if any fermentation. Wolf and Harris,[5] and Hall [3] reported the production of gas and amino acids in several mediums. Sordelli,[8] and Wolf and Harris found considerable production of butyric and acetic acids, although other investigators found little or no change in the acidity of mediums during the growth of the organism.

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[1] Compt. rend. Soc. de biol., 1915, 78, p. 274.
[2] Compt. rend. Acad. d. sc., 1916, 163, p. 449.
[3] Hall, I. C.: Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 501.
[4] La Gangrene Gazeuse, 1917, p. 318.
[5] Jour. Path. & Bacteriol., 1918, 22, p. 1.
[6] Compt. rend. Soc. de biol., 1920, 83, p. 1369.
[7] Jour. Bacteriol., 1925, 10, p. 321; Footnotes 2, 5, 8 and 9.
[8] Compt. rend. Soc. de biol., 1922, 87, p. 838.

The growth of dense white balls or white crystals after 4 days or more has been reported by a number of writers, who assumed the product to be tyrosine.[9] Hall and Finnerud [10] examined the crystals and substantiated the guess of previous workers.

The proteolytic activity of Clostridium histolyticum is much more rapid in vivo than in vitro. Nasta [11] has shown the presence of an active extracellular protease in fresh dextrose broth cultures.

The evolution of NH_3 and CO_2 is considered to be accomplished by means of enzymes acting on the protein or amino acid substrates. Such reactions are generally not specific for a particular amino acid, so that tyrosine, $HOC_6H_4CH_2CHNH_2COOH$, is generally destroyed during putrefaction, along with the other amino acids. There is no apparent reason why tyrosine should not be destroyed with the other nitrogenous constituents in cultures of this particular organism, except that it is less soluble, crystallizing out from solution. A few other organisms exhibit the same phenomena. Further work may show that the destruction of amino acids is more specific than present observations would indicate.

It was thought that by a quantitative study of the tyrosine content of some proteins before and after bacterial growth any considerable destruction of tyrosine would be detected. It was also thought that a study of the tyrosine content of the various food proteins would be valuable in itself because of the great discrepancies in results previously published, and the recognized importance of tyrosine for proper nutrition.

EXPERIMENTAL WORK

A pure culture of Clostridium histolyticum was secured from the Hygienic Laboratory in Washington, D. C. This was used for all inoculations after transplanting into sterile broth. There was no evidence of contaminating organisms during the growth of cultures used for the final analysis.

Preparation of Mediums.—Mediums were prepared by mixing protein material and nutrient broth to contain approximately 20% solids. These were inoculated with a loopful of broth inoculated with the organism mentioned. Controls were prepared at the same time which were not inoculated. One set of inoculated material was incubated at 37 C., and one at approximately 15 C. Observations were also made on cultures kept at room temperature. Anaerobic conditions were secured by a layer of refined petroleum oil added before sterilization.

Previous work has shown that the organism grows over a wide P_H range [12] and that the P_H may change during growth. Consequently in this work no

[9] Henry, H.: Jour. Path. & Bacteriol., 1922, 21, p. 370. McIntosh and Feldes: Med. Res. Council Special Rept., 1917, No. 12. Ibid., No. 39.

[10] Proc. Soc. Exper. Biol. & Med., 1921-22, 19, p. 48.

[11] Compt. rend. Soc. de biol., 1922, 87, p. 279.

[12] Jour. Bacteriol., 1919, 6, p. 419.

effort has been made to control or study changes in acidity. The large amount of protein matter present in the mediums used would act as an adequate buffer, at least until marked growth was secured.

The organism grows aerobically, but better anaerobically. It will grow in the cold and at room temperature, but the optimum growth is at 37 C.

TABLE 1

Cultural Characteristics

Mediums	Time in Days for:			General Notes
	Noticeable Growth	Noticeable proteolysis	Appearance of Tyrosine (Approx.)	
Edestin (2 gm. with 100 c c. broth)	3	5	40	Complete proteolysis
Casein (22 gm. with 78 c c. broth)	7	15	40 Medium production	Gas; marked digestive action
Salmon (75 gm. with 154 c c. broth)	2	12	40 Medium production	Gas; pellicle formation; medium digestive action
Tuna (75 gm. with 154 c c. broth)	2	12	40 Abundant production	Pellicle formation; medium digestive action
Beef (200 gm. with 157 c c. broth)	4	15	6 Very abundant at end of 40 days	First turned red; marked digestive action
Milk (200 c c.).....	4	[illegible]	Not distinct	Gas; white particles, perhaps casein, separated out; decided brown and white layers at end of growth period
Cottage cheese (158.5 gm. with 141.6 c c. broth)	10	50	12 Medium production	Green; marked proteolytic action
Blood (8 c c. with 22 c c. broth)	7	30	45 Abundant production	Gas; complete proteolysis
Cheese (200 gm. with 212 c c. broth)	6	Not marked	60 Slight production	Pink coloration; pellicle formation; white amorphous substance separated out
Gluten (14 gm. with 200 c c. broth)	3	5	40 Abundant production	Green; decided proteolysis; slight odor
Peptone (2.5 gm. with 50 c c. broth)	7	—	40 Abundant production	Pellicle formation

No analyses were made of the mediums incubated at 15 C. as crystals were just beginning to form at the time of starting the analytical work. However, the growth was characteristic: pellicle formation, gas production, and coloration, approximately like those of the mediums incubated at 37 C.

At room temperature proteolytic action occurs rapidly, with the separating out of tyrosine crystals in all the mediums studied.

Table 1 shows the growth characteristics of cultures incubated at 37 C. There was considerable blackening of mediums after incubation, but little odor was noticed.

Analytic Procedure.—Several methods are available for the estimation of tyrosine in protein material. Although much of the work done up to the present time has been done gravimetrically,[13] the results obtained by this method are generally considered too low. The bromination method[14] has been little used. The use of Millon's reagent[15] has been reported on favorably, with certain modifications, since this investigation was under way, but the older procedure was not dependable.

The method used in this work was essentially that given by Folin and Denis.[16] In its application there is some interference from other products of proteolysis, notably tryptophane. The results obtained by this procedure at least meet the requirements for comparative work, and have been used in many investigations. The method of Folin and Looney[17] will be checked against the present method at a later date, but from preliminary investigation, the results will probably not differ greatly from those given in this paper.

The method of procedure for the mediums made up with broth was to autoclave at 120 pounds' pressure for 20 minutes. This served to dissolve crystalline material and to kill the bacteria. While hot this was filtered with suction, and the residue dried and weighed.

After filtering, duplicate 1 gm. samples of the dried solid residue were weighed out and hydrolyzed for 24 hours under an air condenser with 25 c c. of 20% HCl. The total filtrate was measured and duplicate 25 c c. samples of this were hydrolyzed with 25 c c. of 20% HCl. After hydrolysis, the solutions, both of solid and filtrate, were filtered and the filtrate made up to 100 c c. Aliquot parts of this were added to 5 c c. of phosphotungstic-phosphomolybdic reagent. After five minutes, 25 c c. of saturated Na_2CO_3 were added. A blue color developed in from one half to three quarters hours. At the same time a standard tyrosine solution was made up by taking 5 c c. of tyrosine solution (1 mg. tyrosine) and treating in the same way. After the maximum color had developed, the solutions were compared with the standard in a Kober colorimeter.

As a further blank test, the foregoing procedure was carried through with dry commercial proteins.

DISCUSSION

From a study of table 2 it is evident that there is a great variation in the degree of tyrosine destruction. In the peptone, beef, gluten, casein, tuna, edestin and cheese mediums, all of which showed distinct growth and digestive action, a considerable portion of the tyrosine was destroyed. In the salmon, cottage cheese and milk mediums there was practically no destruction of tyrosine, although growth and tyrosine separation were

[13] Plimmer: Chemical Constitution of the Proteins, Monograph, Pt. 1, p. 17.
[14] Ibid., p. 22.
[15] Fürth and Fischer: Biochem. Ztschr., 1924, 154, p. 1.
[16] Plimmer: Chemical Constitution of the Proteins, Monograph, Pt. 1, p. 20.
[17] Jour. Biol. Chem., 1924, 51, p. 421.

similar to the others. It is interesting to note that, with the exception of cottage cheese, the greatest amount of tyrosine was destroyed in the mediums showing marked digestive action.

The extent of hydrolysis of the proteins digested is shown by comparing columns 4 and 5 in table 2. The cheese, edestin, milk and beef mediums indicate that all the protein taken into solution was hydrolyzed to such a degree that all the tyrosine present reacted with the color reagent. In the other cases also considerable tyrosine was found to be present previous to acid hydrolysis.

TABLE 2

Results of Analysis

Protein Material§	G. Tyrosine per 100 gm. Original Dried Material							
	Controls			Inoculated Material				
	1 Filtrate After Hydrolysis	2 Solid Residue	3 Total 1 + 2	4 Filtrate Before Hydrolysis	5 Filtrate After Hydrolysis	6 Solid Residue	7 Total 5 + 6	8 Difference 3 — 7
Cheese*	—	3.18	3.18	0.21	0.21	1.03	1.23	1.95
Edestin*	—	4.64	4.64	4.84	3.89	0.19	4.08	0.56
Salmon	0.44	1.54	1.98	0.35	0.78	1.29	2.07	—0.09
Tuna	1.73	2.68	4.41	0.28	0.59	1.72	2.31	2.10
Cottage cheese	0.23	2.51	2.74	0.61	1.64	1.13	2.77	—0.03
Casein†	—	6.23	6.23	0.84	1.31	0.14	1.45	4.78
Milk‡	0.20	—	0.20	0.15	0.18	—	0.18	0.02
Gluten	0.66	3.98	4.64	0.22	3.16	0.01	3.17	1.47
Beef*	—	3.39	3.39	0.23	0.15	0.33	0.48	2.91
Peptone	—	4.84	4.84	0.59	4.01	—	4.01	0.83

* Control analyses were made on the original solid material.
† Broth was completely absorbed; no filtrate.
‡ The figures for total tyrosine on milk are based on the whole milk and not on the solid content.
§ During the standardization of the procedure for analysis, gliadin, edestin and casein were analyzed with the following results: 4.07, 4.15, 4.15; 4.46, 4.47; 5.12, 5.11, 4.99, 5.10 % tyrosine, respectively.

It seems probable from a general survey of the results that there is a period of proteolysis during the early growth of the organism during which there is little destruction of amino acids. The separation of tyrosine would vary greatly with differences in the P_H of the mediums, as shown by Hitchcock's studies on its solubility.[18] Consequently, different mediums would not be expected to show uniform separation of tyrosine parallel with proteolysis.

[18] Gen. Physiol., 1924, 6, p. 747.

In previous investigations the time for hydrolysis of proteins has generally been 12 hours. Our results show that for dry beef a longer period was necessary. After a brief study of the time of hydrolysis in relation to tyrosine production, it was found that 24 hours was preferable to a longer or shorter period.

The identity of the white crystaline deposit was established by examination of its solubility, microscopic crystal structure, melting point, Millon's reaction, Folin's reaction, and the xantheoproteic reaction.

SUMMARY

A quantitative study was made of the tyrosine content of edestin, salmon, tuna, cheese, casein, milk, gluten, beef, cottage cheese and peptone before and after growth of Clostridium histolyticum. The relation of the digestive action of the organism to the formation of tyrosine crystals in the medium was noted. In 7 of the 10 mediums, destruction of tyrosine was found. In the remaining 3 cases there was practically no destruction of tyrosine during approximately 3 months' growth of the organism.

FURTHER STUDIES ON A DIPLOCOCCUS FROM MEASLES

PREVENTION OF MEASLES BY IMMUNE GOAT SERUM

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In previous articles, one [1] of us has described a filter-passing, gram-positive green-producing, diplococcus which was isolated from the blood in special mediums, almost constantly in the preeruptive and early eruptive stage of measles. This organism has constantly been found in smear preparations and cultures from the respiratory tract during this same period. Specific agglutinins, opsonins and complement fixing bodies for this coccus have been demonstrated in the blood of patients with measles as the symptoms subside. The same symptoms and lesions produced in monkeys, rabbits and guinea-pigs by infective measles material were also caused by this organism, while other bacteria isolated from the same plates failed to produce the measles reactions. Rabbits successfully inoculated with the measles diplococcus showed no reactions when reinoculated with fresh measles material. The diplococcus isolated from the blood and lungs of rabbits inoculated with measles cocci also produced Koplik spots and rashes when injected into normal rabbits. Intracutaneous tests with killed anaerobic cultures of this diplococcus in rabbits and human beings appeared to produce skin reactions which are specific for measles.

Since these experiments indicated that this coccus might be of etiologic significance in measles, it seemed worth while to produce immune serum with this organism in large animals.

Others have shown that animals recovering from measles produced experimentally with measles virus are immune to reinfection; Blake and Trask [2] demonstrated this for monkeys; Duval and D'Aunoy,[3] for guinea-pigs; and Grund,[4] Scott and Simon,[5] for rabbits. Scott and

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[1] Tunnicliff, R.: Jour. Am. Med. Assn., 1917, 68, p. 1028; Jour. Infect. Dis., 1918, 22, p. 402; Jour. Am. Med. Assn., 1918, 71, p. 104. Tunnicliff, R., and Brown, M. W.: Jour. Infect. Dis., 1918, 23, p. 572. Tunnicliff, R.: Ibid., 1919, 24, pp. 76 and 181. Tunnicliff, R., and Moody, W. B.: Ibid., 1922, 31, p. 1. Tunnicliff, R.: Ibid., 1925, 37, p. 193.

[2] Jour. Exper. Med., 1921, 33, p. 385.

[3] Ibid., 1922, 35, p. 257.

[4] Jour. Infect. Dis., 1922, 30, p. 86.

[5] Am. Jour. Hyg., 1925, 5, p. 1.

Simon also showed that the blood of rabbits infected with measles virus will protect uninoculated rabbits when later infected with measles material.

Degkwitz [6] mentions immunizing large animals with cultures which he thinks contains the cause of measles, but no details are given.

Nicolle and Conseil,[7] Degkwitz,[8] McNeal,[9] Weaver and Crooks,[10] Zingher [11] and others have proved the protective power of convalescent measles serum against measles. Since monkeys, guinea-pigs and rabbits were found suceptible to the green-producing diplococcus isolated from patients with measles, it seemed possible that goats might also react to this organism, and if so the blood drawn during convalescence might show protective power against measles. A goat was chosen rather than a horse on account of the danger of sensitization with horse serum if also used in diphtheria and other diseases and also on account of the general impression that goat serum is not toxic to man.

A young brown goat received intravenous injections of a 10 c c. suspension in salt solution of green-producing measles diplococci. The organisms were grown 24 hours in tubes containing 10 c c. of ascites dextrose broth, centrifuged and suspended in salt solution. Six tubes were inoculated, 2 tubes each with diplococci from the throat, from the blood and from the nose, respectively. The injection was followed by a rise of temperature of 1 F. for most of the time from the 4th to the 13th day. The leukocytes rose from 11,600 to 15,600 the day after the injection, returning to normal the following day. The number dropped below normal from the 6th to the 14th day; the lowest counts were 6,400 on the 9th and 10th days. There was a decrease in the number of amphophils. There was a slight conjunctivitis on the 9th day. The mucous membrane of the mouth became very congested from the 8th to the 14th day. No Koplik spots were observed. An eruption was not seen, but the skin of the goat was so dark that a rash could have been observed only with difficulty.

Agglutinins for the 3 strains of diplococci injected developed in the serum in low dilutions as the temperature subsided.

The goat was bled on the 18th day following the injection. Aerobic and anaerobic cultures of the blood were sterile. Tricresol, 0.3%, was added to the serum. The serum of this goat convalescing from the reaction produced by the measles diplococcus was tested to determine whether it would protect rabbits against infective material obtained from human and in one case from rabbit measles.

The rabbits were carefully shaved on the sides and sufficient time allowed for them to recover from any irritating effect of shaving before the measles material was injected.

A rabbit received intratracheal injections of 5 c.c. of a 24-hour ascitic broth culture of the washings of the nose and throat of a patient with measles,

[6] Deutsch. med. Wchnschr., 1924, 50, p. 374.
[7] Bull. et mém. Soc. med. d. hôp. de Paris, 1918, 42, p. 336.
[8] Ztschr. f. Kinderh., 1920, 27, p. 171.
[9] Jour. Am. Med. Assn., 1922, 78, p. 240.
[10] Ibid., 1924, 82, p. 204.
[11] Ibid., 1924, 82, p. 1180.

taken on the 1st day of the eruption. Four days after inoculation the rabbit showed Koplik spots persisting 3 days, and a rise in temperature of 2 F. from the 5th to the 7th days. On the 7th day a well marked maculopapular eruption appeared. Four c c. of the citrated blood of this rabbit were injected intravenously into each of the two rabbits that had been injected subcutaneously 3 days before with 5 cc. immune goat serum; into one rabbit injected previously with 5 cc. normal goat serum, and into one uninoculated rabbit. The rabbits receiving the immune goat serum were protected, showing no rise in temperature, no Koplik spots, no eruption. The rabbit receiving injections of normal goat serum showed a rise of 1.5 F. of temperature on the 7th day and a slight maculopapular eruption. The untreated rabbit had a rise of 0.6 F. of temperature on the 7th day, a Koplik spot from the 5th to 7th day and an erythema. This eruption persisted from the 8th to the 11th day, and was followed by desquamation.

In the next experiment, 4 c c. of salt solution washings from the nose and throat of 2 patients with Koplik spots and the beginning of eruptions, were injected intratrachcally into 2 rabbits 24 hours after the subcutaneous injection of 5 c c. immune goat serum; into 1 rabbit receiving a similar injection with 5 c c. of normal goat serum, and into 1 rabbit receiving no serum injection. The 2 rabbits receiving the immune goat serum showed no reactions. The rabbit receiving normal goat serum showed Koplik spots on both sides of the mouth on the 11th and 12th days after innoculation, a well marked maculopapular eruption on the 11th and 12th days, followed by pigmentation and desquamation. The third rabbit had a rise in temperature of 1.3 F. on the 11th day and a faint eruption on the 12th day.

Seven rabbits were now injected subcutaneously, four with 5 c c., one with 2 c c., one with 1 c c. of immune goat serum and one with 5 c c. of normal goat serum. The following day each of these and a fresh untreated rabbit received intravenously 3 c.c. of citrated blood from a patient with Koplik spots and a well marked eruption. The rabbits injected with immune goat serum showed no reactions. The rabbit receiving normal goat serum had a rise of 1 F. of temperature on the 4th day, with a faint rash from the 8th to the 12th days, followed by desquamation. The untreated rabbit showed a rise of 1 F. on the 8th day, Koplik spots on the 8th to the 11th day, but no eruption.

The serum of this goat was tested to determine whether it could neutralize killed measles diplococci when injected into rabbits. Equal parts of a 24-hour anaerobic ascites dextrose broth culture, killed with 0.5% phenol, were mixed in respective lots with salt solution, normal goat serum, convalescent human measles serum and the immune goat serum; after incubation at 37 C. for 3 hours, 0.2 c.c. of each mixture was injected intracutaneously into the sides of a shaved normal rabbit. Within 24 hours, the mixtures of antigen with salt solution and normal goat serum produced bright red, circumscribed, indurated areas 1 cm. in diameter, while the mixtures of antigen with convalescent human measles serum and the immune goat serum produced no reactions.

These experiments show that the serum of a goat convalescent from the injection of green-producing measles diplococci protected 10 rabbits against the subsequent inoculation of infective measles material from human and rabbit measles. Three rabbits receiving injections with normal goat serum and 3 rabbits that did not receive any injections of serum showed all or some of the symptoms characteristic of measles in rabbits —a rise in temperature, Koplik spots and eruption.

By intracutaneous tests in normal rabbits, the serum of the goat injected with measles cocci was shown to neutralize killed measles diplococci as effectively as did convalescent human measles serum, while normal goat serum had no such effect.

Since it is often difficult to obtain sufficient convalescent human measles serum for immunization, and since these experiments indicated that the serum of a goat convalescing from the injection of measles diplococci protected rabbits against measles, the serum of the goat was tried in a few human cases, convalescent human measles serum, collected by T. T. Crooks, being used as a control.

George Sutherland inoculated 9 children who had not had measles, 5 with 5 c c. of convalescent human measles serum and 4 with 4 c c. of convalescent goat serum. The serum was injected one or two days after exposure to measles. In each group one child had a typical attack of measles on the 12th day, the other children not developing measles.

O. J. DeWitz injected two children, one with 5 c.c. of convalescent human measles serum and one 5 c c. of the goat serum on the 5th day after exposure to measles. Both children had modified attacks of measles.

At the Children's Memorial Hospital, 16 children who had not had measles previously were inoculated, 5 with 5 c c. of convalescent human measles serum and 11 with immune goat serum, 6 of these receiving 2 c c., the others 5 c c. The exposure of the 6 infants receiving 2 c c. of goat serum was doubtful. The remaining children receiving goat serum were injected on the day of exposure. Three children receiving the human serum developed typical measles on the 2d, 9th and 16th days after injection. The child developing measles on the 2d day received the serum too late to expect any protection. Two children injected with goat serum developed measles on the 10th and 12th days. The remaining children showed no signs of measles. In no instance was there any indication of sensitization with this goat serum. In one immune adult, the goat serum produced an area of urticaria 5 inches in diameter at the site of inoculation, 6 days after injection.

A sufficiently large number of cases were not immunized to draw any conclusion, but the results of these experiments suggested that the convalescent goat serum was as effective as the convalescent human measles serum in preventing measles. Neither serum was fresh; the goat serum was 6 months, the human serum 9 months, old. It was thought that possibly larger doses of goat serum should be used on account of the slight reaction in the goat to the measles organisms.

Since the experiments with the goat serum appeared to protect rabbits and human beings against measles, another goat was immunized with the same strains and in the same manner except that one tube of inoculated broth was also injected. This goat was white. The reaction was similar to that in the other goat. The injection of the organism was followed by a leukocytosis for 3 days, during which time the goat was sick. On the 10th day after injection, there was a slight leukopenia, with an increase in the number of lymphocytes. On this day there was congestion of the conjunctiva and mucous membranes of the

mouth, which lasted for 5 days. On the 13th day, the goat was sick and had diarrhea. On the 14th day, a macule was observed on the lip. The temperature rose 3 F. on the third day, remained above normal for one more day and then dropped to normal (101.5 F.) until the 13th day, when it rose 2 F. and remained above normal for 5 days. The goat was bled on the 20th day. Green-producing diplococci grew in the anaerobic culture, and therefore this serum could not be used. Blood collected 7 days later was sterile; 0.3% of tricsol was added to the serum.

This serum was tested for its neutralizing power on killed measles diplococci as in the previous experiment. The antigen diluted with salt solution in 24 hours produced a circumscribed, red, indurated area 2 c c. in diameter; the antigen mixed with normal goat serum produced a slightly smaller reaction, while the mixture with immune goat serum and antigen showed no reaction.

This serum was first used 2 weeks after collection; 5 c c. injected intramuscularly into an adult person, who was slightly sensitive to a previous injection of goat serum, within 8 hours produced an area of redness and edema 12 inches in diameter at the site of injection, but caused no other symptoms.

At the Children's Memorial Hospital 6 susceptible children each received 8 c c. of this goat serum 1 or 2 days after exposure. Five children were protected; 1 developed measles on the 13th day. Five other children in this group, who did not receive goat serum, developed measles. Three children, 2 of them patients of J. H. Skiles, receiving 8 c c. of the serum on the 5th day after exposure, and 2 children injected on the 3rd day after exposure, developed measles. Two children, however, who were injected on the 2d day after exposure were protected. An infant receiving 6 c.c. of serum did not develop measles, but exposure was doubtful.

At the Michael Reese Hospital, Eloise Parsons tried this serum in 2 children; 2 others received convalescent human measles serum, and 2 were not immunized. Although they all gave negative histories of measles and all were exposed, none developed measles.

At the Presbyterian Hospital in the service of C. G. Grulee, 8 children with negative histories of measles given goat serum, did not get measles, but the history of exposure was doubtful.

The ages of the children in these experiments varied from 6 months to 12 years, and did not appear to influence protection. The children who were protected were from 3 to 8 years old.

Of the 24 children injected with the second goat serum, 15 were sensitive and developed urticarial eruptions of varying intensity. None showed any systemic reactions. After preservation for 8 months this serum appears to have lost its toxic effect.

The experiments with the serum of the second immune goat serum showed that by intracutaneous tests in normal rabbits, it could neutralize the action of killed measles diplococci, while normal goat serum had no such effect. Of 8 susceptible children receiving the serum on the 1st and 2d days after exposure, 1 developed measles, 2 injected on the 3rd day and 3 on the 5th day, all contracted measles. These results indicate that this goat serum had some protective power against measles, but not sufficient to prevent measles if injected after the first 2 days after exposure.

Although immune goat serum has been used in too few cases to draw any conclusions as to the prevention of measles, we think that further investigation is warranted, especially with serum of more highly immunized goats.

SUMMARY

The serum of a goat convalescing from a reaction produced by the inoculation of green-producing measles diplococci protected rabbits against a subsequent injection of infective material from measles. The rabbits injected with normal goat serum and those untreated showed some or all of the characteristics of measles in rabbits—a rise of temperature, Koplik spots and an eruption.

Convalescent goat serum protected human beings against measles as effectively as convalescent human measles serum when injected on the 1st and 2d days after exposure to measles.

By intracutaneous tests in normal rabbits, the serum of goats injected with diplococci from measles, as well as convalescent human measles serum, were shown to neutralize the action of killed measles diplococci, while serum of normal goats had no such effect.

The serum of one immune goat was found toxic to nearly all persons injected with it, but after 8 months preservation it appears to have lost its toxic effect.

While these experiments do not furnish conclusive evidence of the protective value of immune goat serum, they warrant further investigation.

MORPHOLOGIC VARIATIONS OF BACTERIA IN THE LAG PHASE

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The phenomena of growth, senescence and rejuvenescence in bacteria have been the subject of considerable study and speculation. Since these phenomena are apparently essentially identical with those exhibited by higher organisms, such studies, relatively easily made and rapidly completed, will probably prove of more and more importance in experimental biology.

The rate of growth of bacteria, as expressed by one or another form of curve, shows a regular variation from the time of seeding until the culture is dead; after remaining dormant for a period, the cells increase in number with increasingly greater rapidity until a maximum rate is reached, then with decreasing rapidity until the number remains stationary, after which the cells begin to die with increasing rapidity until a maximum death rate is attained, then with decreasing rapidity, some cells remaining viable in most cultures for long periods. Most authors who have written on the growth curve have found it convenient to divide this curve into phases.

Lane-Claypon [1] recognized 4 phases: a latent period of no growth or slow growth, a period of maximum growth, a stationary period, and a period of death. This division was also followed by Penfold [2] and by Chesney.[3] Buchanan [4] further subdivided these phases, making 7 in all: the initial stationary phase, the lag phase (or phase of positive acceleration), the logarithmic growth phase, the phase of negative acceleration, the maximum stationary phase, the phase of accelerated death, and the logarithmic death phase.

Most interest has centered in the first stage, generally referred to as the lag phase. This is usually defined as the period from the time of seeding until a maximum growth rate is attained; but Buchanan points out that this contains 2 periods, a preliminary stationary phase when no growth occurs, the organisms lying dormant, and a phase of steadily

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[1] Jour. Hyg., 1909, 9, p. 233.
[2] Ibid., 1914, 14, p. 215.
[3] Jour. Exper. Med., 1916, 24, p. 387.
[4] Jour. Infect. Dis., 1918, 23, p. 109.

increasing growth rate until a maximum is reached. He applies the term "lag phase" to this second period of positive acceleration. A number of factors influencing the duration of the lag phase have been determined. It has been found to vary, as would be expected, with the species or strain of organism, with the nature of the medium, and with the temperature of incubation. But keeping these factors constant, it has also been found to vary with the size of the inoculum, being more prolonged with smaller seedings (Penfold), and with the age of the parent culture, being absent when the latter is in the maximum growth phase (Barber,[5] Penfold, Chesney).

A number of theories have been proposed to explain this lag phase. These have been well reviewed by Penfold and Buchanan. Some of them are but slight variations of others, and they may be well considered in four main groups.

The toxic theory, maintained by Chesney, looks on the latent period as the time necessary for the organisms to recover from an injury sustained in the previous culture. It must be assumed, then, that the cessation of growth in a culture is always due to the accumulation of toxic products of metabolism, but this is not the case, with the exception of a few organisms. It is well known that streptococci and some anaerobes may be inhibited by the acid which they produce, since growth will continue longer if the medium is neutralized. The pneumococcus with which Chesney performed the experiments on which his theory was based, has recently been shown to produce hydrogen peroxide in quantities perhaps sufficient to explain his results[6]; but with the majority of organisms no such toxic substances can be demonstrated. In all of Eijkman's[7] experiments in which he claimed to have demonstrated a growth inhibiting substance, this substance was not separated from the bacteria, and the results may be just as well explained by an excess of organisms in the medium. Conradi and Kurpjuweit[8] claimed to have separated this hypothetic toxic substance from the bacteria by dialysis, but I have repeated some of their experiments with several species of bacteria with entirely negative results. The recent observations of Graham-Smith[9] seem to show conclusively that cessation of growth is due to a partial exhaustion of the medium, since the addition of further nutrient material to a culture which has ceased growing leads to a renewal of growth.

We may designate as metabolic theories a second group which postulate that growth can reach a maximum rate only when certain products have been elaborated by the organisms and have accumulated to an optimum concentration. These may be end products of metabolism or intermediate substances in the synthesis of protoplasm, substances similar to vitamines, or substances of the nature of enzymes. Since the lag phase is shortened by increase in the size of the seeding it would seem that the cells mutually stimulate each other,

[5] Jour. Infect. Dis., 1908, 5, p. 379.
[6] Morgan and Avery: Jour. Exper. Med., 1924, 39, p. 331.
[7] Centralbl. f. Bakteriol. Abt. I, 1904, 37, p. 436; 1906, 41, p. 367.
[8] München. med. Wchnschr., 1905, 52, p. 1761.
[9] Jour. Hyg., 1920, 19, p. 134.

and therefore the enzyme, if it exists, must be extracellular. "This mutual acceleration of the multiplication-rate of contiguous cells has been designated the 'allelocatalytic effect'" by Robertson,[10] who believes that at each cell division some of the endocellular autocatalyst is liberated into the medium, and the accumulation of this extracellular autocatalyst leads to a progressive increase in the growth rate. Sherman and Albus,[11] however, failed to find any lag in cultures inoculated from a culture in the maximal growth phase even if the inoculum was reduced to 1 cell per cubic centimeter; while Cutler and Crump,[12] and Greenleaf[13] failed to demonstrate the "allelocatalytic effect" in cultures of protozoa, and Peskett[14] had similar negative results with a yeast.

Theories based on adaptation and selection may be conveniently considered together in a third group. Penfold suggested that transfer from one medium to another may cause delay in initiation of growth because the organism must adopt itself to the new medium, but he dismisses this theory because transfer to the same medium was accompanied by lag. This objection, however, is hardly valid, since undoubtedly in the course of growth the organisms change the composition of the medium in which they are growing. I have had an opportunity to test this theory on a strain of B. coli which had been subcultivated in parallel cultures continuously for five years on standard beef extract agar and in a synthetic medium composed of nutrient salts and glycerol. Both of these sub-strains were inoculated simultaneously into beef extract broth and this synthetic medium, taking care to keep the volume of medium and the size of the seeding nearly equal. There was no marked difference in the degree of lag exhibited in the four cultures. It would seem therefore that adaptation is not of prime importance. Penfold also suggested that "the inoculum consists of organisms having individually different powers of growth and that during the lag the selection of a quick growing strain occurs in response to some selecting agent in the peptone." This would explain the gradual acceleration in rate of growth but would not account for the sometimes long period of complete dormancy. Penfold objected to this theory, because he found that parallel cultures even with very small seedings showed the same degree of lag, but I shall present some evidence to support this explanation.

Finally, Buchanan proposed a theory which has much to support it. He suggests that at the cessation of growth bacteria go into a resting state, and when transferred to a new medium they must undergo a process similar to germination before they can start to grow; i. e., that even in non-spore-formers there occurs a physiologic process analogous to spore formation and spore germination, and that the latent period is occupied by this process of germination. Such physiologic differences between the cells of young and old cultures of bacteria have been demonstrated by Sherman and Albus,[15] who found the young cultures much more sensitive to injurious agents, such as cold, heat, and salt solution. In a later paper,[16] they showed that this physiologic transformation occurs previous to active cell proliferation, and suggest that the latent period is the time required for the transformation or rejuvenescence. Buchanan

[10] Chemical Basis of Growth and Senescence, 1923.
[11] Abstr. of Bacteriol., 1924, 8, p. 6.
[12] Biochem. Jour., 1923, 17, p. 878; 1925, 19, p. 450.
[13] Proc. Soc. Exper. Biol. and Med., 1924, 21, p. 405.
[14] Biochem. Jour., 1924, 18, p. 866; 1925, 19, p. 474.
[15] Jour. Bacteriol., 1923, 8, p. 127.
[16] Ibid., 1924, 9, p. 303.

also believed that the cells did not all respond to the new medium simultaneously, but that some "germinated" sooner than others, thus explaining the gradual acceleration in growth rate.

Clark and Ruehl [17] first described morphologic differences between the cells from young cultures and those from older ones. In a series of quantitative studies of these variations, recently summarized,[18] I have obtained some data which seem to indicate that the cells of bacteria undergo a regular metamorphosis during the growth of a culture, and that during the early stages of growth the cells are analogous to the embryonic cells of a multicellular organism. I shall review here such of this work as seems to throw some light on the nature of the lag phase. My observations have been made largely on Bacillus megatherium, but I have also worked with Bacterium coli, a diphtheroid bacillus, and the cholera vibrio.

The early stages of growth with its accompanying morphologic variations may be followed in developing microcolonies on slide cultures. A loopful of culture is stirred in a tube of molten and cooled agar, and a generous loopful of this agar is spread on a flamed coverslip which is then placed on a hanging drop slide smeared with petrolatum and containing a drop of water. The slide is placed in a stage incubator; a cell or group of cells is located with the medium power lens and then examined continuously with the oil lens. I have watched the development of a number of such colonies of B. megatherium. Generally I have found that single isolated cells failed to grow, or exhibited such a prolonged latent period that I lost patience and discontinued observation before growth commenced. With a short chain growth begins earlier.

In fig. 1, I have presented a series of semidiagrammatic camera lucida drawings of such a developing microcolony. It will be observed that during the first 3 hours practically no changes in morphology are apparent; the size remained fairly constant; one cell disintegrated, another became deformed by constriction at one end. With the initiation of active proliferation, the cells undergo a change, becoming larger and less granular. In this particular colony, the increase in size of cells and the increased rate of cell division became apparent at the same time, but in some others I have noted an increase in size and disappearance of granules some time previous to any cell division. It will be noted that not all of the cells became active, a group of three remaining dormant throughout the period of observation.

[17] Ibid., 1919, 4, p. 615.
[18] Henrici: Science, 1925, 41, p. 644.

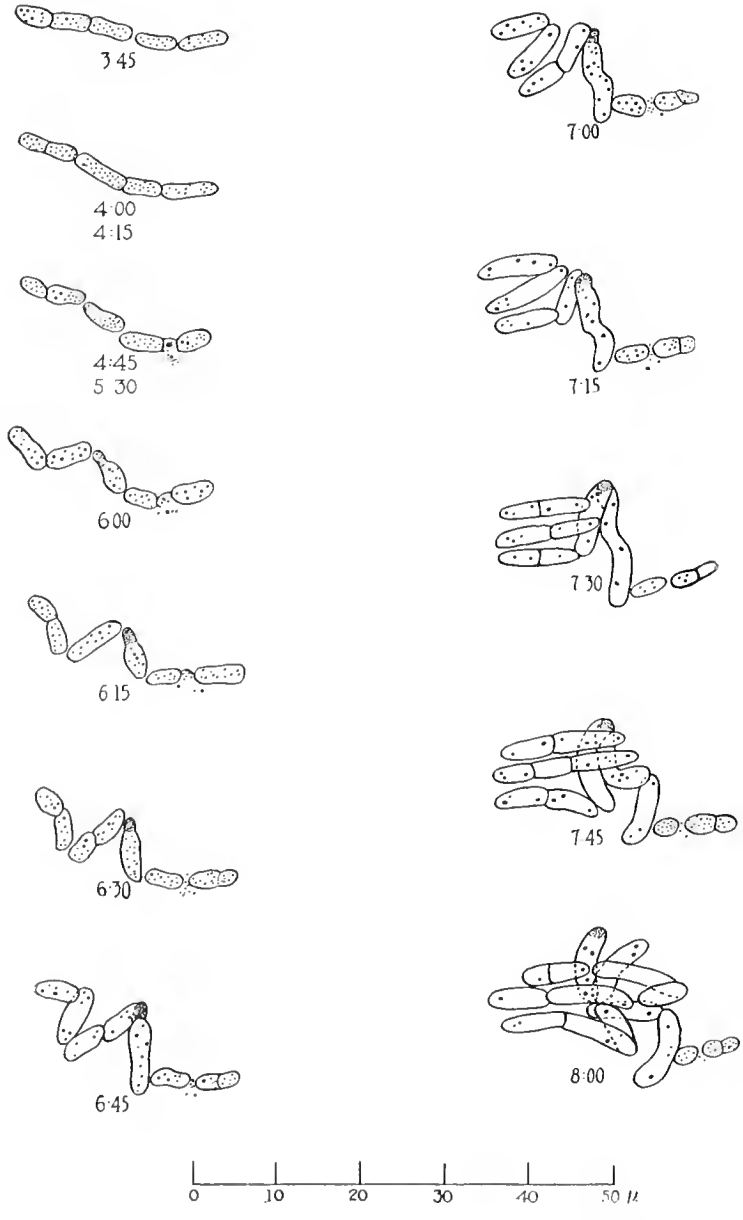

Fig. 1.- Semidiagrammatic camera lucida drawings of a developing microcolony.

It is apparent that, since the cells change in size while growing, the number of cells is not a true measure of growth, which could only be obtained by computing the volume of protoplasm. This may be done roughly by obtaining the sum of the lengths of the organisms, since they

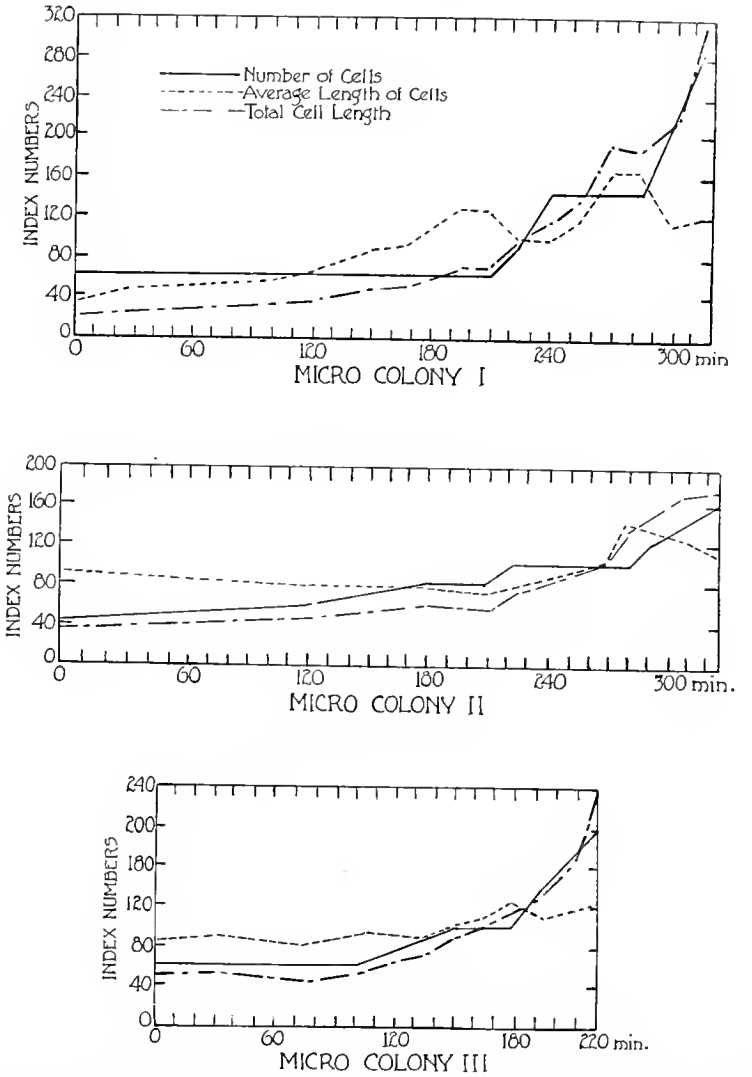

Fig. 2.—Curves for the number of cells, the average length of the cells and the sum of the lengths of the cells of three different microcolonies of B. megatherium, the third one being the colony illustrated in figure 1.

do not increase so much in thickness. Fig. 2 shows curves for the number of cells, the average length of the cells and the sum of the lengths of the cells of 3 different microcolonies of B. megatherium, the third one being for the colony illustrated in fig. 1. In order to plot these

different values to the same scale they have been converted to index numbers, the index in each case being the average value for the entire period of observation.

The first colony developed from 2 cells which grew about equally and divided synchronously. The cells began to increase in size almost at once, the rate of increase showing a gradual acceleration. Preceding each cell division the size remained constant for a short period. The rate of increase was still greater after the first division, and the average size of the cells at the time of the second division was considerably greater than at the first. While there were no cell divisions for more than 3 hours, the curve for total cell length showed an accelerating increase from the beginning of the observation.

The second colony developed from a group of two cells, one of which was slowly dividing at the time the preparation was made; only one of the daughter cells resulting from this division grew; the other two cells decreased somewhat in size and failed to divide as long as observation was continued. As a result the average length of the cells dcreased slightly during the latent period.

The third of these microcolonies has already been described.

From these microcolonies a number of facts may be learned. The increase in number of cells is accompanied by an increase in size. This is apparently more marked with a small seeding (first colony starting from 2 cells) than with a larger seeding (third colony developing from 5 cells). The latent period before beginning cell proliferation is not entirely occupied by a transformation of the small (mature) cells to large (embryonic) cells; there is also a lag phase in the curves for cell size. Not all of the cells respond equally to the new medium, some remaining dormant for considerable periods of time; there does actually occur, therefore, a selection of a rapidly growing strain in some cases. The cells which remain dormant may continue to change in morphologic characters as they would have had they remained in the old medium, becoming smaller in size, distorted in form, or dying and disintegrating.

Since there is such a difference in the rate at which different cells respond to the new medium there must be considerable difference (as has been demonstrated) in the curves for cell size and number of cells in separate microcolonies started from only a few cells. We may also determine these values for tube cultures by removing samples from the culture at regular intervals, counting the number of cells and measuring the size or form of the cells. This has been done a number of times with Bacillus megatherium.

The counting of the cells has been done by direct microscopic methods either using a hemocytometer or from stained slides by a modification of the method of Breed and Brew, which I have previously described.[19] The measure-

[19] Henrici: Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 293.

ment of the cells has been accomplished by projecting slides negatively stained with Congo red, generally at a magnification of about 3,000 diameters, the length of the cells being determined by means of dividers. From 200 to 500 cells have been measured from each sample.

In general the same results were obtained as with the microcolonies. The curves for cell size show a lag phase not quite as long as the latent period in the curves for number of cells, the increase in size preceding slightly the increase in numbers. This lag phase is absent if the cells were in the embryonic form in the parent culture, present if they were in the small or resting form. If the stage of dormancy is prolonged, the cells may become still smaller for a period after inoculation, i. e., they react as they would have had they remained in the parent culture. The degree to which they do so varies with the size of the seeding,

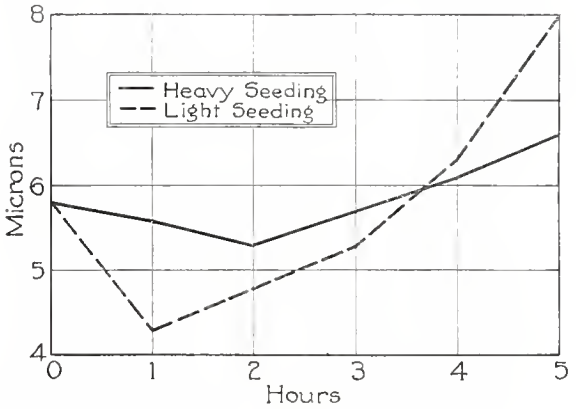

Fig. 3.—Curves for length of cells in two parallel broth cultures, one being inoculated with one-tenth as many cells as the other.

being greater with smaller seedings. This is shown in fig. 3, which presents curves for the length of the cells in two parallel broth cultures, one being inoculated with a tenth as many cells as the other.

This phenomenon is not peculiar to B. megatherium. As I have described in an earlier paper,[20] the mature forms of a diphtheroid bacillus are large, the embryonic forms are small. With this organism the cells were found to become distinctly larger during the latent period. In a study, recently published,[21] of morphologic variations of the cholera vibrio, I have also pointed out that the cells reacted during the lag phase as they would have if they had remained in the parent culture.

Most interesting results were obtained when the frequency curves for the distribution of the cells according to size at different stages of growth were compared.

[20] Ibid., 1922, 20, p. 179.
[21] Henrici: Jour. Infect. Dis., 1925, 37, p. 75.

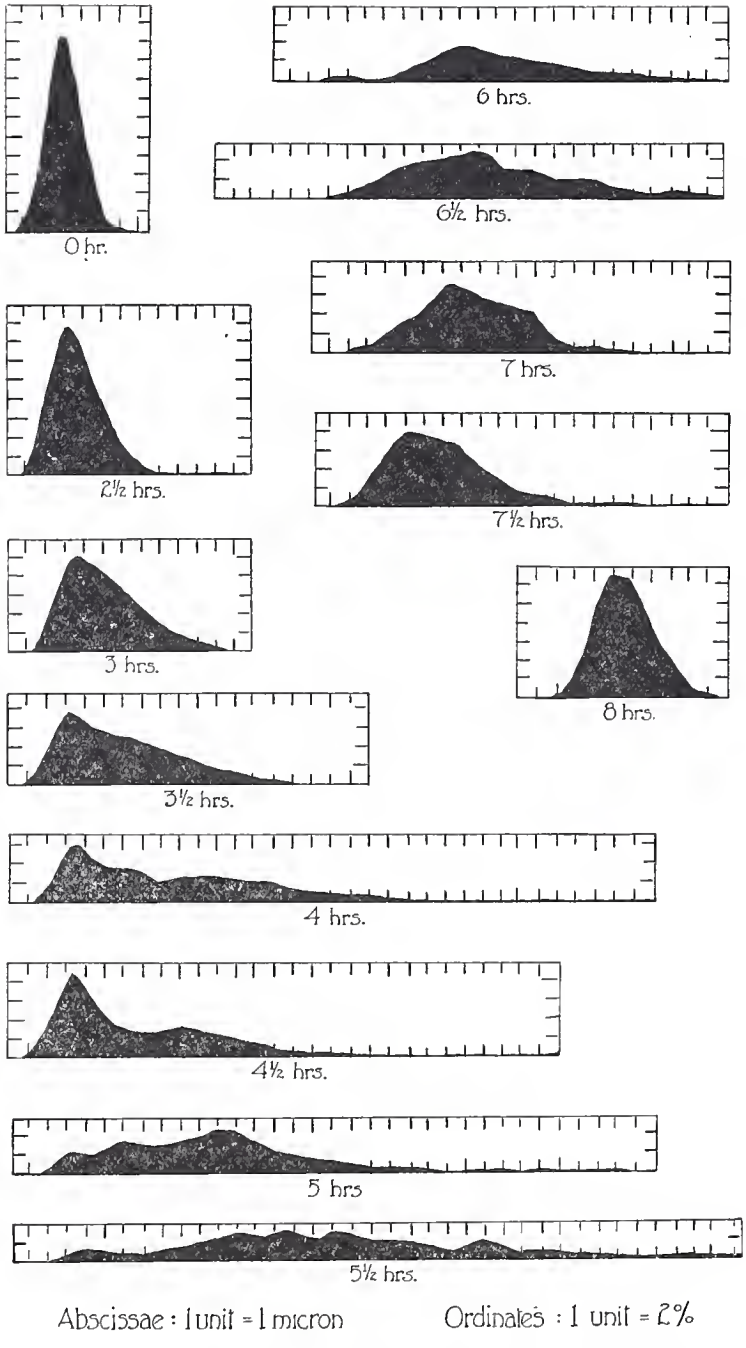

Fig. 4.—Frequency curves for distribution of cells according to size at different stages of growth.

Such a series of curves is presented in fig. 4. These were determined from an agar slant culture of B. megatherium which commenced to show active growth (in numbers) after about 3 hours, reached its maximum rate at 6 hours, and had practically stopped growing at 12 hours. The statistical constants for these curves together with the cell counts at the same time intervals are given in the accompanying table.

The curves show a regular and progressive change in form reaching its maximum at 5½ hours, followed by a gradual change to the original form which was complete at 10 hours. As the cells increased in size, they also increased in variability in size, as indicated by increased range and dispersion; but, more than this, the curves become increasingly

TABLE 1

RATE OF GROWTH

Hours of Growth	No. of Cells per C.Mm.	Average Length, Microns	Mode, Microns	Median, Microns	Standard Deviation	Coefficient of Variation
0	..	3.4	3.0	3.1	0.80	23
½	90	...	...	...	....	..
1½	110	...	...	...	....	..
2	139	..	...	...	....	..
2½	178	3.9	3.5	3.5	1.38	35
3	196	5.3	4.0	4.7	2.20	42
3½	209	6.5	3.5	5.5	3.58	55
4	244	9.1	3.5-4.0	10.2	5.55	61
4½	416	7.3	3.5	5.4	4.70	64
5	1,370	11.3	11.0-12.0	10.6	6.16	54
5½	2,080	19.8	14.5-15.0	16.8	8.67	43
6	5,920	13.0	10.5	12.8	4.42	34
6½	10,380	15.0	14.0	14.2	5.92	37
7	15,040	9.0	7.5	8.4	3.12	34
7½	18,540	7.2	5.5	6.5	2.89	32
8	21,080	5.7	5.0	5.3	1.40	24
8½	38,409	5.0	4.0-4.5	4.4	1.87	36
9	57,240	4.3	3.5	3.7	1.98	46
9½	79,700	4.7	3.5	3.7	1.49	32
10	63,200	4.5	4.0	4.0	1.02	23

The number of cells per cubic millimeter has been calculated on the basis that the entire growth was suspended in 2 c c. of water, the actual quantity used having been varied according to the heaviness of growth. The averages, standard deviations, and coefficient of variation have been computed from the observed values. The modes have been determined by inspection from the smoothed curves, and the medians have been obtained from the smoothed cumulative percentile curves.

skewed or asymmetrical, and at the 4 hour period the curve becomes definitely bimodal, the cells being divided into a large group of small cells and a small group of large cells. In the subsequent periods this new group rapidly grows at the expense of the old one. The curves for the 5 and 5½ hour periods are quite irregular, probably because the number of cases (500) was not sufficient for such highly variable material; but the 6 hour curve shows clearly the establishment of a now large group of large cells with a very small remaining group of small cells which completely disappear during the next half hour. After this period the curve undergoes a series of changes leading to a gradual

return to the original form; the range becomes more restricted, the curve becomes more symmetrical, and moves as a whole in the direction of smaller size.

The same types of changes in frequency distribution have occurred in all of the other cultures of this organism which have been examined, though they have never been so pronounced as in the case here presented, which was a culture lightly seeded. In every case the curves presented marked skewness during the period of accelerating growth. Bimodality has always appeared when the inoculum was small, and has disappeared when the seeding was increased in quantity. The degree of skewness has also been greater with smaller seedings. A single observation with Bacterium coli showed skewness without bimodality.

It is clear from these curves that not all of the inoculated cells commence to increase in size at once, for if they did so, and all increased proportionately, the curve would simply move in the direction of greater size without changing its form. But if at first a few and then increasingly greater numbers of cells commenced to grow (and elongate), as Buchanan has suggested, there would be produced just such a series of changes in distribution as have been described, increased range and dispersion, and skewness; but as long as this process was continuous, while there would be produced a gradual upset of the whole curve with the final development of a new mode, there would not occur distinct bimodality such as is apparent in this series of curves. If, however, out of the inoculated cells one group started to grow while the others remained dormant (as was seen to occur in microcolony III), bimodality with the new mode growing at the expense of the old one would result.

The influence of the size of the seeding on the distribution of the cells can also be explained on the basis of this variation in "growth potentiality," i. e., power to respond to the new environment by increase in size and by cell division. The larger the number of cells in the seeding, the greater will be the continuity in this variation in growth potentiality. Thus, if we plant only 2 cells, they may grow either at the same rate or at dissimilar rates; because of the tendency to variation in living matter the chances are in favor of the latter possibility. If we plant 3 cells, the chances will be in favor of 3 rates: a maximum, a medium, and a minimum. The larger the number of cells inoculated, the greater will be the number of gradations in growth rate between the maximum and minimum. With a very large number of cells presenting an evenly graded series, growth accompanied by increase of size in the

growing cells will cause the frequency curves to show the increased range and skewness noted. With very small seedings, there will be greater chances for gaps to occur between the maximum and minimum, with the development of bimodality. In such cases, then, the selection of a rapidly growing strain from the inoculum, as has been suggested by Penfold, actually occurs.

SUMMARY

1. The initiation of growth in a culture is accompanied by a morphological transformation of the mature or resting cells to embryonic or growing cells.

2. From the evidence here presented it would seem that the lag phase as usually defined, i. e., the time from the moment of seeding until the maximum growth rate is attained, is composed of two parts: an initial dormant phase and a succeeding phase of accelerating growth during which apparently different factors are operative.

3. The period of dormancy is not entirely occupied by a transformation of the mature cells to the embryonic type. The curves for cell size may also show a lag phase, dependent on the age of the parent culture.

4. During the period of dormancy the cells may continue to change for a time in the same way that they would have had they remained in the parent culture. The degree to which they do so varies with the size of the seeding, being greater with smaller seedings.

5. The inoculated cells show considerable variability in the rate at which they respond to the new environment by a morphologic transformation, as indicated by the increasing skewness in the frequency curves for size of cells during the accelerating growth phase. This variability probably explains the gradual acceleration in growth rate. In some cultures the frequency curves may show bimodality indicating a selection of a rapidly growing strain from the inoculum. The degree of skewness and tendency to bimodality varies with the size of the seeding, being greater with smaller seedings.

RELATIONS OF SURFACE TENSION TO BACTERIAL PHENOMENA

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It is now a well established fact that changes in the physical conditions under which bacteria grow may markedly affect their activities. The effects of variations in temperature, concentrations of various ions, osmotic pressure and numerous other factors have been investigated. There is one physical force, however, which has not been adequately studied, namely, the force that arises from the mutual attraction between molecules and manifests itself as surface tension. Bacteriologists are coming more and more to the belief that an explanation of many hitherto little understood phenomena may rest on a knowledge of the action of surface forces. According to MacCallum,[1] "surface tension is the most powerful and most important of all forces concerned in the life of animal and vegetable cells."

THEORETICAL DISCUSSION

The rôle of surface tension in cell life is not a simple one. It may therefore be helpful in forming our ideas on this point to consider some of the more important changes that can be wrought by surface forces in the physical and chemical state of objects and substances.

Of the many phenomena which result from the action of surface forces, probably the most important and best understood is adsorption. Gibbs[2] has described this mathematically. His formula concerns the relation of surface tension reducents to the surfaces of their solutions, and is based on the law of entropy. This law requires that any change that tends to reduce the amount of free energy in the universe shall tend to take place. Surface tension is a form of free energy. If a substance reduces surface tension, it must tend to carry the reduction to a maximum. This may be accomplished in several ways. For example, the actual extent of the surface may be reduced. Should this be impossible, as in the case of solid or rigid bodies, the reducent can

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[1] Science, 1910, 32, p. 449.

[2] Scientific Papers, 1906, 1, p. 56.

lower the amount of free energy to the greatest extent only by accumulating at the surface of these bodies. This accumulation is known as adsorption, and may be selective, chemical, or altered by the electrical charges present. The nature of the adsorption depends on the character of the surface and substances and other factors involved. For instance, heat may accelerate the speed but diminish the extent of adsorption, while viscosity will retard the rate but not the extent. It has been shown that most substances which have been adsorbed on a surface are much concentrated, and that in many cases their solubilities increase. It is probable that many gases are actually liquefied when adsorbed. It is clear that such condensations and increases in solubility must, from the law of mass action, greatly facilitate chemical reactions. For example, if two substances, such as oxygen and formic acid, are adsorbed on the surface of a third inactive substance, such as finely divided platinum, we have a reaction facilitated, if not entirely controlled, by surface forces. The platinum does not enter into the reaction, but remains to adsorb more oxygen and more formic acid, and so continue the process. In such a reaction the platinum acts as a catalytic agent, and its action as such apparently depends on its large surface development. In a simple system of this kind we can predict, from a knowledge of the substances and surfaces involved, what the result of a given combination will be.

But let us consider a more complicated state of affairs. Let the particles of inert platinum be replaced by minute globules of protein, each bounded by a membrane, of unknown composition, having unknown properties.

By the term membrane we must understand in this connection that in many cases this may be formed merely by the condensation (adsorption) of material, at a surface serving to separate two phases (Alsberg,[3] Chambers [4]).

Imagine a number of such globules suspended in a solution, not of oxygen and formic acid, but of a number of substances ranging in complexity from simple ions like $Na+$ and $H+$ to unstable compounds like the conjugate proteins. Enclose a small quantity of the suspension in a semipermeable envelop, and we have something resembling a cell. This is indeed a complicated and highly reactive system. Here we have an interplay of surface forces, osmotic pressure, electrical

[3] Science, 1911, 34, p. 97.
[4] Proc. Soc. Exper. Biol. & Med., 1922, 20, p. 72.

energy, chemical affinities and other factors, the resultant of which is life, and the chemical and physical possibilities of which seem boundless.

It seems reasonable to suppose, on the basis of such work as that of Fisher and Hooker,[5] Bredig,[6] and others, that enzymes are catalytic agents, dependent for their action on extensive surface development. By analogy then, a view of the cell as a sort of catalytic unit does not seem unreasonable. Wells [7] reviews many observations of which the only possible explanation "seems to be that the enzyme effects are brought about by surface phenomena."

In addition, the possibility of affecting intracellular pressure by treating such cell suspensions as bacterial cultures with surface tension alterants must be considered.

LITERATURE

In addition to theoretical considerations, there are experimental data that indicate that surface forces play an important rôle in cell activities. The work of Rhumbler,[8] Butschli,[9] Kisch,[10] Czapek,[11] MacCallum,[12] Billard and Dieulafe,[13] and many others might be referred to.

However, most of the work cited has been done with protozoa or the cells of higher plants and animals. Regarding the bacteria, we are not nearly so well informed. Comparatively speaking, bacteriologists have only recently begun to realize that many of the phenomena which they study may be the direct results of, or at least greatly influenced by, the presence of substances that reduce surface tension.

For instance, Avery,[14] while working on a selective medium for the cultivation of B. influenzae, made use of the observation that soaps prevent the growth of certain bacteria. No mention of the part that surface tension might play in this action is made, and the chemical effects were apparently the only ones considered.

Engering [15] devised a medium for use in roughly differentiating between the group of Corynebacteria. His medium contained sodium oleate, but the rôle of surface tension in the action of the medium was not considered. In view of work to be described it is believed now that whatever efficicacy Engering's medium may possess is dependent on the low surface tension induced by the sodium oleate present in it.

[5] Jour. Lab. & Clin. Med., 1918, 3, p. 373.

[6] Ergebn. Physiol., 1902, 1, p. 134.

[7] Chemical Pathology, 1920, p. 50.

[8] Ergebn. Physiol., 1914, 14, p. 474.

[9] Investigations on Microscopic Forms and on Protoplasm, Trans. E. A. Minchin, 1904.

[10] Biochem. Ztschr., 1912, 40, p. 152.

[11] Ueber eine Mtehode zur direkten Bestimmung der Oberflaschenspannung der Pflanzenzellen, 1911.

[12] Proc. Roy. Soc., 1913, 86, p. 527.

[13] Compt. rend. Soc. de biol., 1904, 56, p. 146; 1906, 61, p. 323.

[14] Jour. Am. Med. Assn., 1918, 71, p. 2050.

[15] Centralbl. f. Bakteriol., I, O., 1922, 89, p. 120.

Brooks,[16] in a study of the effect of various substances on the respiration of B. subtilis, found that the effects of two surface tension reducents were so different that she could not believe that surface tension played any part in the observed results. This conclusion she based on certain evidence that saponin, one of the substances involved, produced its effect by penetration of the cell membrane, and that as a result, its effects were probably not due to surface action. Theoretically it might appear that if a substance could penetrate the cell membrane, it would come into more intimate contact with the intracellular colloids and therefore have a greater effect, because of its ability to alter surface tension, than otherwise. Saponin, however, has peculiar physical properties which may complicate an interpretation of Brooks' results.

Calmette,[17] although not particularly interested in surface tension effects, used bile (an effective depressant) in his experiments with the tubercle bacillus. He found that bovine tubercle bacilli when subcultivated a number of times on potato-bile-glycerol medium became avirulent and could safely be used for immunizing purposes in young cattle. It is suggested that some of the results obtained by Calmette were due to the lowered surface tension which the bile might have induced.

Reasoner[18] showed that solutions of toilet and shaving soaps were speedily fatal to T. pallida. This, of course, is in harmony with the results of other workers who have used lysis by surface tension reducents as a basis of differentiation among the spirochetes. These methods are of interest because of the very low surface tensions that probably prevail due to the presence of considerable quantities of soap or bile in the medium or solutions used.

Larson, Cantwell and Hartzell[19] were probably the first to make a direct study of the effects of surface tension on the growth of bacteria. They found that pneumococci and streptococci were, in general, inhibited by surface tensions below about 50 dynes. They also observed that the growth of B. anthracis was suppressed by surface tensions below 46 dynes, and that sporulation of B. subtilis was inhibited by low surface tensions. The rôle of surface tension in pellicle formation was studied by Larson and Larson.[20] In 1923, Larson and Montauk[21] cultivated tubercle bacilli in a medium containing soap and glycerol and found that the organisms grew in this medium with a diffuse cloudiness, and appeared to have lost all pathogenicity, giving rise merely to local and temporary nodules on injection into guinea-pigs. Tubercle bacilli in sputum seemed to lose their pathogenicity when treated with sodium ricinoleate. These results lend plausibility to the suggestion that low surface tension may have been the important factor in Calmett's experiments.

Ayers, Rupp, and Johnson[22] made a study of the effect of surface tension on the final P_H value attained by cultures of streptococci in dextrose-infusion medium. They suggest the possibility of using this as a practical means of differentiating between various types of streptococci. Their measurements of growth are based on the production of acid. Acids, however, hydrolyze the types of soaps used by them for reducing purposes. Interpretations of their results would obviously be complicated by these changes in the amount of reducent present as well as by the products of the reactions. In addition, the

[16] Jour. Gen. Physiol., 1921, 3, p. 527.
[17] Ann. de l'Inst. Pasteur, 1921, 35, p. 561.
[18] Jour. Am. Med. Assn., 1917, 68, p. 973.
[19] Jour. Infect. Dis., 1919, 25, p. 41.
[20] Ibid., 1922, 11, p. 407.
[21] Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 229.
[22] Jour. Infect. Dis., 1923, 33, p. 202.

organic acids, esters and other substances produced by the streptococci as a result of their action on glucose have been shown to have, of themselves, a profound effect on surface tension. The presence of these substances might, of course, change the character of the experiments entirely. Furthermore, it would seem that acid production may not be a reliable measure of growth since some strains of streptococci, form large quantities of acid from a given sugar after comparatively little growth and other strains very little acid after extensive growth. Experimental work is in progress in this connection.

An interesting development of our knowledge of certain defensive mechanisms of the body is found in the work of Welch[23] and Klotz[24] and new data given later on in this article.

Welch, in 1890, described the appearance of rows of pneumococci in the fluid of empyema following pneumonia. The pale staining capsular substance was to be seen and occasionally one or more deeply staining cocci, the remainder of the cocci in the row having wholly or partly disappeared. The mechanism by which the pneumococci were destroyed was not described at the time.

In 1905, however, Klotz demonstrated the presence of soaps in inflammatory exudates. The presence of these soaps was believed to be in some way connected with the defensive action of the host, but the mechanism of this action was not suspected.

Because of certain results to be reported now, it is believed that the appearances described by Welch were produced by the soaps demonstrated by Klotz and that the mechanism involves the action of surface tension largely if not exclusively.

The rôle of surface tension in the mechanism of disinfection, while not closely related to the subject at present under discussion, nevertheless introduces an important principle which deserves mention here. This is connected with the formation of such toxic substances as acids, esters, aldehydes, etc., by bacteria in mediums containing fermentable carbohydrates. That many of these substances are powerful surface tension reducents is known. The rôle of surface tension in augmenting the poisonous action of a substance already toxic has been discussed by Leonard and Frobisher.[25]

In this connection the work of Walker[26] is of interest. Walker has shown that not all soaps have the same effect on the same organism in a given concentration and that a given soap may be more effective against one organism than against another. He points out that the action of soaps as bactericidal agents is probably enhanced by their ability to lower surface tension. He believes that the germicidal action of soaps and the resistance of bacteria may later be correlated with surface tension.

But it is true that some soaps lower the surface tension more than others in the same concentration and may therefore be more effective germicides. Indeed, Berczeller has shown that germicidal activity of a substance is closely related to, if not entirely dependent on, its ability to lower surface tension. In view of these considerations, the possibility that bacteria which are sensitive to low surface tension may inhibit their own growth by the production of toxic surface tension reducents in culture mediums need hardly be indicated.

While many valuable studies have been made on certain problems in the field, no general experimental review has been attempted, with the

[23] Bull. Johns Hopkins Hosp., 1890, 1, p. 73.
[24] Jour. Exper. Med., 1905, 7, p. 663.
[25] Jour. Urol., in press.
[26] Jour. Infect. Dis., 1924, 35, p. 587.

possible exception of the work of Larson and his associates, my studies were undertaken to provide a foundation with points of departure for future investigations.

GENERAL METHODS USED AND EXPERIMENTS WITH MEMBERS OF THE COLI-TYPHOID GROUP AND THE STAPHYLOCOCCI

This section deals with the general methods of securing data on surface tension and describes a series of experiments with coli-typhoid organisms and staphylococci.

Surface Tension Measurements.—The surface tension measurements were made with a du Noüy tensiometer.[27] This instrument measures the pull on a platinum ring of the surface of the liquid under investigation. The pull is transmitted through a lever fixed at right angles to a twisted steel wire, the torsion of which is registered on a dial.

The method of making the surface tension determinations deserves detailed description because deviations from a method fixed on may, at times, introduce a considerable error. It was shown by du Noüy[28] that the surface tension of a solution such as serum which contains surface tension reducents becomes continuously lower on exposure to air. If readings are made at intervals during a period of several hours, each successive reading will be lower than the preceding. The change is ascribed to an accumulation of molecules of the reducents at the surface during the time of the experiment. This appears to be a general property of solutions of organic substances with large molecules, and has been confirmed by Gouwens[29] and observed also by me. In all cases, therefore, every reading has been made after the lapse of an arbitrary period of two minutes after placing the fluid under investigation in a watch glass.

In drawing conclusions from measurements of surface tension one should keep in mind certain limitations of the method. The air-fluid interface is the only place where, with the means at hand, we may conveniently measure the surface tension of the fluid. This interface may be very different from the cell-fluid interface, in its measurable surface tension and in the physical and chemical properties of the layers of fluid immediately adjacent to the cell. In other words, it is a recognized possibility that a substance may exert some influence at the cell-fluid interface which are not manifest in measurements made at the air-fluid interface.

All of the determinations were made at a temperature of 20 C., using 1 c c. quantities of the fluids. Aqueous solutions were used throughout unless otherwise noted.

Measurements of Growth.—In the experiments with the coli-typhoid group measurements of growth were made by ordinary plate dilution methods. For the measurements of the growth of all other organisms studied a "loop" method was adopted which may be considered as a modification of Breed's[30] method of counting bacteria in milk. A similar "loop" method has been found valuable

[27] Jour. Gen. Physiol., 1919, 1, p. 521.
[28] Jour. Exper. Med., 1922, 35, p. 575.
[29] Jour. Infect. Dis., 1922, 31, p. 237.
[30] Centralbl. f. Bakteriol., I, O., 1911, 30, p. 337.

by Miller[31] for estimating the quality of milk. The modification described here, while not an exact enumeration, gave the desired information as to whether there was a large or small or moderate number of organisms present.

Ordinary microscopic slides were ruled off into 5 mm. squares. After shaking the tubes sufficiently to distribute all the growth, the desired material was carefully spread, one standard loopful to one square. These slides were dried slowly on a level surface and stained directly with Loeffler's methylene blue. Twenty or 30 fields in different sections of each smear were scrutinized, and an estimate made of the amount of proliferation that had taken place. Proliferation in the control tube was used as a basis of comparison. Excellent growth, in which every field had hundreds of organisms in it, was designated as "4 +," lesser degrees of proliferation as "3 +," "2 +," and "1 +." Where doubt existed as to whether any proliferation had taken place, and whether the few scattered organisms that could be found after careful search were the result of growth, or were remnants of the original inoculum, a ± was used. Where no organisms were found a minus sign (—) was used.

Preparation of Glassware.—Except in the more exact preliminary studies, or when noted elsewhere, all glassware used was well boiled in soap solution, and thoroughly rinsed in hot tap water and then in distilled water.

Reagents to Lower Surface Tension.—The first series of tests consisted of the comparison of the surface tension depressing power of sodium oleate, sodium ricinolate, sodium glycocholate, bile and saponin. A 1% solution of each reducent, except bile, was prepared in triply distilled water. Bile was used undiluted. The triply distilled water had been distilled once from a block tin still, and twice from pyrex glass apparatus. A quantity of strongly alkaline potassium permanganate solution was added to the distillation flask before the last distillation and the first 200 c c. of this distillate were discarded.

Graded quantities of the solutions and bile were added to 10 c c. quantities of triply distilled water and to like quantities of 0.5% peptone-water. The surface tension of each mixture was determined and curves made, platting the surface tensions against the amounts of depressant used (chart 1). In connection with certain irregularities in some of these curves, it is interesting to note the experiments of Buglia.[32] Buglia's quantities and reagents were similar to those used in the foregoing determinations. The irregularities in his curves also correspond in a general way with those noted in chart 1. The information gained from these determinations facilitated subsequent surface tension adjustments.

Saponin had been recommended as an effective surface tension reducent. It gives marked evidence of being a reducent in its property of frothing when its solutions are shaken. But it has the peculiarity, as shown by Ramsden,[33] of accumulating at surfaces so rapidly and so extensively as to form rigid membranes. When attempts are made to measure the surface tension of its solutions, irregular results are obtained. By measuring directly the surface tension of various solutions of saponin, it was shown to be an ineffective reducent for our purposes; nor could any considerable or regular reduction be obtained by adding quantities of these solutions to pure water. Furthermore, it was observed that when froth was produced on the surface of solutions of saponin, the bubbles would assume irregular elliptical shapes. These observations led to the belief that the substance besides being an ineffective reducent, was unusual in some of its physical properties, and might give confusing results.

[31] Jour. Lab. & Clin. Med., 1924, 9, p. 3.
[32] Biochem. Ztschr., 1909, 22, p. 1.
[33] Proc. Roy. Soc., 1904, 72, p. 156.

For these reasons saponin was not used in the main growth experiments, although it was included in the list of substances which were tested for poisonous properties.

Poisonous Properties of Reducents.—Should any of the reagents used for lowering surface tension exert an inhibitory effect on bacteria, it would, of course, be necessary to know before interpreting the result whether the inhibition were due to the lowered surface tension per se or to some chemically poisonous, activity of the reagent. For this reason, the substances which had already been shown to be effective in lowering surface tensions were tested as to their poisonous properties. A number of substances were included in this series of tests which had not been previously tested as surface tension reducents. While these additional substances had heretofore been regarded as either ineffective reducents or as probably too poisonous for use in connection with bacteria, they were included in this series partly for the sake of completeness and partly to check certain other experimental data.

Graded amounts of sterile ethyl alcohol (99%); isoanyl alcohol (C.P.); ethyl acetate (C.P.); butyric acid (C.P.); 1% solutions of sodium oleate (Merck); sodium glycocholate (Merck); a soap made from castor oil; saponin (Merck), and pure, fresh ox bile, were added to 5 c c. quantities of sterile 0.5% peptone-water. The tubes containing the mixtures were inoculated with B. typhosus from an agar slant. A control tube containing only the peptone water was also inoculated. The surface tension of each culture was determined and the tubes then incubated at 37 C. for 24 hours. Note was then made of the tubes in which growth had taken place.

There seems to be little relation between the ability of these reducents to kill or inhibit B. typhosus and their ability to reduce surface tension. For example, it was observed that alcohol, while only reducing surface tension to about 65 dynes, nevertheless prevented growth in concentration of 1%, whereas bile did not inhibit, and probably aided, growth in the same concentration, although it reduced the surface tension to about 44 dynes. This experiment showed that sodium oleate, sodium ricinoleate, sodium glycocholate and bile are effective surface tension reducents and that B. typhosus was able to grow well in their presence. Iso-amyl alcohol and butyric acid were shown to be effective reducents, but B. typhosus did not grow in medium containing any considerable quantity of them. Butyric acid changes the reaction of the medium to such an extent that it would obviously complicate interpretations based on surface tension alone.

Ethyl alcohol and ethyl acetate were shown to be ineffective reducents for the purposes of this study. The former was shown to be not only a poor reducent, but also to have the property of preventing growth of B. typhosus in very low concentrations. Saponin as a reducent, has already been discussed. It is apparently not an effective reducent for the purposes of these studies.

Nutritional Properties of Reducents.—Some information was desired as to the possible nutritional properties of the reducents, since they were to be considered, as far as possible, as bland physical reagents.

Mixtures were made in specially cleaned tubes, of 10 c c. of triply distilled water, and suitable quantities of the selected reducents. The quantities of reducent were based on the data given in chart 1, and were selected as the minimum amounts of each reducent that produced a maximum reduction of surface tension. Each mixture was inoculated with 1 cc. of a 1 : 500,000 dilution, in water, of a 24-hour, 0.5% peptone culture of B. aerogenes, which was selected as being the most active, metabolically of the 3 organisms of the group under investigation. It was at first supposed that if B. aerogenes were unable to metabolize the reagents or their impurities, the other organisms would also be unable to do so. This point was subsequently checked for all 3 organisms. It was at first assumed that in 1 c c. of the diluted culture used for inoculum there would be so little peptone added to the mixture as to be a negligible factor in the ensuing growth. A control containing only the distilled water was also inoculated. After inoculation, the surface tension and P_H of each mixture was determined, and the tubes were incubated at 37 C.

It was found that B. aerogenes grew practically as well in the bile mixture as in the peptone control. Growth in the castor oil soap mixture and the glycocholate mixture was sufficient to indicate that these reagents must contribute something toward the growth of B. aerogenes. The growth in the sodium oleate mixture was very small; some increase in numbers took place in the plain water control. This is not surprising in view of the work of Savage and Wood,[34] Hinds,[35] and Winslow and Cohen [36] and the well known difficulty of producing absolutely pure water in the laboratory.

The experiment was repeated, using in addition to B. aerogenes, cultures of B. typhosus and B. morgani. These were in all respects like the culture of B. aerogenes just described, except that, in order to remove peptone from the inoculums, they were centrifuged twice, and the bacteria resuspended in sterile triply distilled water. Sodium recinoleate was omitted because, being a soap, its chemical effect, if any, would be similar to that of the sodium oleate and because it was found not to be as strong a reducent as the latter. An examination of table 1 shows that when dissolved in pure water the reducents do not support any considerable growth of B. typhosus or B. morgani. B. aerogenes seems able to multiply to some extent in the glycocholate and oleate solutions. This may be due to the presence of small quantities of impurities known to exist, even in the "C.P." preparations of these substances, which can be metabolized by B. aerogenes.

[34] Jour. Hyg., 1917, 16, p. 221.
[35] Univ. of Ill. Biol. Bull., 14, No. 5, Water Survey Series No. 13, p. 225.
[36] Jour. Infect. Dis., 1918, 23, p. 82.

TABLE 1

GROWTH OF B. AEROGENES, B. TYPHOSUS AND B. MORGANI IN PURE WATER SOLUTIONS OF SODIUM OLEATE AND SODIUM GLYCOCHOLATE. (WASHED ORGANISMS USED FOR INOCULUM)

Organism	Surface Tension Reducent 1%	Quantity of Reducent, C c.	P_H of Mixture	Surface Tension of Mixture Dynes	Bacteria per Cubic Centimeter After				
					0 Hours	16 Hours	24 Hours	48 Hours	72 Hours
B. typhosus...	Oleate	0.5	7.2	37.4	76	200	90	—	3
B. typhosus...	Glych.	0.5	7.2	49.0	85	75	100	—	41
B. typhosus...	None	—	6.9	77.0	85	100	200	—	2
B. aerogenes...	Oleate	0.5	7.2	36.8	616	22,000	250,000	63 Mill.	850 Mill.
B. aerogenes...	Glych.	0.5	7.2	51.1	1,368	250,000	4,720,000	35 Mill.	350 Mill.
B. aerogenes...	None	—	6.9	77.0	1,436	900	500	—	21,000
B. morgani....	Oleate	0.5	7.2	36.8	77	200	200	—	7
B. morgani....	Glych.	0.5	7.2	50.4	95	500	800	—	—
B. morgani....	None	—	6.9	77.0	93	80*	2,500	28 Mill.	18 Mill.

* Loopful of peptone accidentally added.

TABLE 2

EFFECT OF HEATING AND TIME ON THE SURFACE TENSION OF BROTH CONTAINING SODIUM OLEATE

Quantity of 1% Oleate Solution Added to Broth, C c.	Unheated Oleate Solution Added to Broth		Autoclaved Oleate Solution Added to Broth	
	Surface Tensions of the Mixtures, Dynes per Cm.		Surface Tensions of the Mixtures, Dynes per Cm.	
	Before Fractional Sterilization	After Fractional Sterilization	Before Fractional Sterilization	After Fractional Sterilization
	Column 1	Column 2	Column 3	Column 4
0	60.0	60.0	60.0	—
0.1	44.9	44.9	44.9	44.9
0.2	42.9	42.9	42.9	—
0.3	40.9	40.9	40.9	41.5
0.4	—	—	40.2	40.2
0.5	38.8	38.1	38.8	38.1
0.7	37.4	37.4	37.4	—
1.0	36.0	36.0	36.0	36.1
2.0	—	—	33.3	33.3
Surface tension of fresh, 1% oleate solution:				
Unheated			32 dynes	31.3 dynes
Autoclaved			32 dynes	31.3 dynes

Hydrolysis of Soaps.—Another point regarding the use of soaps as surface tension reagents was investigated. Ayers et al., and Larson et al., believe that when soaps are used for the reduction of surface tension there may be an error introduced by hydrolysis of the reagent during incubation. It may be that hydrolysis is brought about by substances produced during bacterial growth, or that a rise in surface tension

occurs because a portion of the soap is removed from the solution by adsorption on dead cells which sink to the bottom of the culture vessel. That any considerable variation occurs spontaneously is shown to be improbable by the following experiments:

A 1% solution of sodium oleate was prepared. Graded quantities of this fresh, unheated solution were added to 10 c c. quantities of sterile, 1% peptone broth in graduated tubes. The surface tension of each mixture was measured, as shown in column 1 (table 2). These mixtures were then sterilized in an Arnold sterilizer for 1 hour on 3 successive days, and the surface tension again measured after making up the loss of water. The results are given in column 2 (table 2).

This experiment was repeated, using, instead of a fresh, unheated solution of oleate, one which had been autoclaved. The results are given in columns 3 and 4, table 2. Finally, the surface tension of both a fresh unheated and an autoclaved 1% solution of oleate was measured before and after sterilization in the Arnold apparatus, with the results shown in the tabulation supplementary to table 2.

It seems that little change in surface tension of such solution occurs as a result of incubation or heat per se when sufficient reducent is used to form a continuous layer of molecules at the surface. This conclusion seems amply supported by the work of McBain.[37] Results of the foregoing experiments with reagents selected for use in surface tension experiments show that:

1. Sodium oleate is an effective reagent and comparatively inert from the standpoint of bacterial metabolism.

2. Sodium ricinoleate is similar to sodium oleate but not quite so powerful a reducent. Solutions are not so turbid as those of oleate.

3. Iso-amyl alcohol is a more powerful reducent than sodium oleate. It is poisonous for some organisms, such as B. typhosus, but not for others, such as B. xerosis. This will be demonstrated later.

4. Sodium glycocholate is only moderately active in lowering surface tension. It may be metabolized by some organisms.

5. Bile is similar to sodium glycocholate.

6. Ethyl alcohol is a fair reducent but poisonous.

7. Ethyl acetate is a rather poor reducent. It is not poisonous in small quantities.

8. Butyric acid is a strong reducent but poisonous.

9. Saponin seems to have peculiar physical properties and is not very effective in lowering surface tension as measured with the du Noüy apparatus.

[37] Third Rept. on Colloid Chem., Brit. Assn. for Adv. of Sc., 1920.

Controls.—In each of the experiments with surface tension a number of reagents of totally unlike chemical composition have been used. It has been assumed that when the same results have been obtained with chemically unlike reagents the effects could be attributed to the physical rather than to the chemical activities of these reagents. Controls on sterility, etc., have been of the usual type. The growth, morphology, etc., of organisms in mediums having low surface tensions has, of course, been controlled by cultivating the same organisms in mediums of ordinary surface tension and using these cultures as bases for comparison.

EFFECTS OF LOW SURFACE TENSIONS ON B. TYPHOSUS, B. MORGANI AND B. AEROGENES

Growth.—Bacilli of the coli-typhoid group were selected for the first experiments because of their wide distribution, the ease with which the organisms may be cultivated and because we believe these bacteria represent a comparatively simple type of organism. B. morgani was selected as intermediate between B. typhosus and B. aerogenes in its metabolic activity. Our first study concerned the growth of B. aerogenes, B. morgani and B. typhosus under conditions of high and low surface tensions.

Twelve tubes were set up. Three contained 10 c c. of 1% peptone solution and served as high surface tension growth controls, one for each organism. This medium was used because of its simplicity, and because, containing little peptone, it had a high surface tension, thus allowing a wide range for experimental adjustment of this factor. Three other tubes contained this medium with the addition of 1 c c. of a 1% autoclaved solution of sodium oleate. Three tubes were also arranged for growth at low surface tension in which the reagent was 1 c c. of 1% autoclaved solution of sodium glycocholate. Three other tubes served as sterility controls for the reagents and medium, respectively.

Each of the growth test tubes was inoculated with 1 c c. of a 24-hour 0.5% peptone culture of the proper organisms, diluted 1 : 1,000,000 in water. Immediately after inoculation the surface tension and P_H of the seeded cultures and sterile controls were measured, and plate counts were made of the number of cells in the inoculum and in each of the tubes in the test. All tubes were then incubated at 37 C., and counts were made after various time intervals.

It is shown in table 3 that in 0.1% peptone, low surface tension has the effect of depressing the growth of these intestinal organisms during the logarithmic period of increase and probably during the earlier phases of growth also. In medium containing amounts of peptone as small as 0.1%, the depression of growth might be ascribed to adsorption of the reducents on the outer surface of the cells or the cell colloids, with a resulting starvation of the organisms. It is possible that this would interfere with the diffusion of the nutrient material into these bodies.

TABLE 3

THE EFFECT OF LOW SURFACE TENSION ON THE GROWTH OF THREE INTESTINAL ORGANISMS

Organism	Surface Tension Reducent*	P_H of Culture	Surface Tension of Culture, Dynes per Cm.	Bacteria After Various Intervals of Incubation †				
				0 Hours	24 Hours	48 Hours	72 Hours	6 Days
B. typhosus	1% Oleate	7.8	34.0	35	7,500	—	420,000,000	1,900,000,000
B. typhosus	1% Glyeh.	7.1	45.6	27	85,000,000	3,620,000,000	5,000,000,000	1,000,000,000
B. typhosus	None	7.2	68.1	30	280,000,000	1,280,000,000	20,000,000,000	11,000,000,000
B. aerogenes	1% Oleate	7.8	34.0	154	1,730,000,000	17,700,000,000	694,000,000,000	1,600,000,000,000
B. aerogenes	1% Glyeh.	7.2	46.3	140	3,020,000,000	12,280,000,000,000	158,000,000,000,000	4,900,000,000,000,000
B. aerogenes	None	7.3	70.1	135	19,640,000,000	—	1,030,000,000,000,000	6,100,000,000,000,000
B. morgani	1% Oleate	7.8	34.0	44	6,200	—	610,000,000	2,100,000,000
B. morgani	1% Glyeh.	7.2	45.6	26	165,000,000	300,000,000	2,000,000,000	1,000,000,000
B. morgani	None	7.2	66.1	35	296,000,000	11,530,000,000	36,660,000,000	90,000,000,000

* 1 c.c. of solution used.
† Per c.c.

A striking similarity between the growth curves of B. morgani and B. typhosus under the various conditions is brought out in table 3. These curves, if superimposed on each other would practically coincide. The following suggestion may be of interest in this connection. We have already discussed the possibility that bacterial protoplasm has a colloidal structure, and that this structure promotes an extensive play of surface forces. There is also evidence that bacterial cells have an investing membrane of definite structure. The fact that the two organisms in question react so similarly to changes in surface tension

TABLE 4

THE EFFECT OF SURFACE TENSION REDUCING SUBSTANCES ON THE GROWTH OF STAPHYLOCOCCI

Surface Tension Reducent	Quantity of Reducent, C c.	P_H of Culture	Surface Tension, Dynes per Cm.	Growth After 48 Hours
1% oleate	0.1	7.0	51.1	++++
1% oleate	0.5	7.1	46.3	++++
1% oleate	1.0	7.1	35.4	++++
1% oleate	1.5	7.3	33.4	++++
1% oleate	2.0	7.4	32.7	+
5% glycocholate	0.1	7.0	43.6	—
5% glycocholate	0.5	7.0	42.9	—
5% glycocholate	1.0	7.1	43.6	—
5% glycocholate	1.5	7.1	44.3	—
5% glycocholate	2.0	7.2	44.3	—
Iso-amyl alcohol	0.01	7.0	59.2	++++
Iso-amyl alcohol	0.05	7.0	48.4	++
Iso-amyl alcohol	0.10	7.0	40.9	+
Iso-amyl alcohol	0.50*	7.0	32.0	±
Iso-amyl alcohol	0.70*	7.0	32.0	—
Ox bile	0.10	7.0	47.7	++++
Ox bile	0.50	7.0	51.2	++++
Ox bile	1.00	7.2	51.2	++++
Ox bile	1.50	7.2	—	++++
Ox bile	2.00	7.3	52.5	++++
None	—	7.0	63.4	++++

* Supersaturated with alcohol.

suggests a similarity in colloidal and membrane structure. It may be that this denotes a closer biologic relationship than has been assigned on the basis of carbohydrate or protein metabolism.

Morphology.—Morphologic changes brought about by low surface tension seemed to be comparatively slight. An extended discussion of the relation of surface tension to morphology will be found in Thompson's "Growth and Form." [38] During the course of the experiments just described, stained preparations were made of the organisms grown

[38] On Growth and Form, 1920.

under conditions of high and low surface tension. It was usually noticed that under conditions of low surface tension (35-40 dynes) the cells tended to become longer and more slender.

As we know (law of entropy), the greater the surface tension of a fluid, the greater the opposition to extension of its surface by the formation of any vacuoles within it. By lowering surface tension, we decrease the opposition to expansion or extension of surface. The fact that these cells do not change in diameter, but do elongate perceptibly, suggests that the rigid cell envelop, if any exists, may not be closed at the ends.

Motility.—The changes observed were rather inconstant and slight. More exact studies must be made before drawing any conclusions on this point. In general, the motility seemed to be somewhat reduced by lowering the surface tension.

EFFECTS OF LOW SURFACE TENSION ON STAPHYLOCOCCI

The general methods used in this series of studies were almost exactly like those described above. The chief variations have been simply in the kind of medium used and the quantities of surface tension reducents added. Of a 24 hour culture in 0.1% peptone (5 c c.) of Staph. albus from the skin, 0.1 c c. was inoculated in the medium described. The results are given in table 4, which shows that for the strain of staphylococcus used iso-amyl alcohol and sodium glycocholate appear to prevent growth because of some property which may not be connected with their power of producing a reduction in surface tension which is measurable at the air-fluid interface. The reduction of surface tension obtained with bile is apparently not great enough to affect the organism, while sodium oleate inhibits growth at about 32 dynes. As a check, a similar experiment was made, using 5 different stains of staphylococci.

TABLE 5

THE EFFECT OF SODIUM OLEATE ON THE GROWTH OF FIVE STRAINS OF STAPHYLOCOCCI

Quantity of 1% Oleate, C c.	Surfaee Tension, Dynes per Cm.	Growth After 48 Hours				
		Strain 1 (sealp)	Strain 2 (albus)	Strain 3 (aureus)	Strain 4 (aureus)	Strain 5 (aureus)
0.05	53.9	++++	++++	++++	++++	++++
0.10	41.0	++++	+++	++++	+++	++
0.50	38.3	++++	+	++	++	+
0.70	32.6	++	..	..	..	+
1.00	30.6	+	+	±	+	+
1.50	30.6	—	—	—	+	±
2.00	30.6	—	—	—	±	±
None	70.2	++++	++++	++++	++++	++++

The data in tables 4 and 5 show that 31 dynes is the lowest surface tension at which any growth of staphylococci occurs, while the surface tension at which good growth is checked is seen to be about 40 dynes. By referring to table 5, it will be seen that the strain of staphylococcus used in the experiment represented by this table failed to grow in the glycocholate mixtures. By repetition of this part of the experiment and by trial with other cultures of staphylococci, it was shown that the staphylococci, in general, were not sensitive to glycocholate, but that the strain in question was unusual in this respect. The strain in question differed from the others in having been subcultivated on agar slants for some months prior to the experiments outlined here.

More interesting, perhaps, than the growth inhibitions were the changes in the state of dispersion of the staphylococci induced by growth in mediums of low surface tension. The clumping of organisms grown in fluids having low surface tensions has been noted by Larson et al., and some relations of surface forces to this phenomenon have been discussed by Green and Halvorsen.[39]

In these studies with the staphylococci, clumping was repeatedly observed in those cultures growing on the low surface tension mediums. The lower the surface tension, the more marked the clumping.

In addition to clumping, an increase in size was observed. It is significant in this connection that we have, along with the clumping and increase in size of many of the cells, a decrease in numbers.

The mechanism and significance of these changes will be discussed farther on.

THE CORYNEBACTERIA

The study of Corynebacteria was undertaken to determine the lowest surface tension at which various members would grow. The surface tension at which vigorous growth dwindled to sparse growth was also sought, since it might have a practical bearing on the preparation of culture mediums. During these experiments it was kept in mind that present methods for differentiation between true diphtheria and pseudodiphtheria bacilli are not satisfactory.

Meader [40] and others have shown that diphtheria bacilli may vary in virulence, morphology and ability to ferment carbohydrates. The same is true of the pseudodiphtheria bacilli. It appears, therefore, that the cultural and biochemical properties mentioned may not be entirely

[39] Jour. Infect. Dis., 1924, 25, p. 5.
[40] Jour. Infect. Dis., 1919, 24, p. 145.

reliable criteria for differentiation. A means of differentiation which does not depend on the production of enzymes or toxins or peculiarities in morphology, all of which vary greatly in this group, should have a distinct advantage. A method which, it is believed, possesses this advantage is described herewith.

The surface tension of an agar prepared according to Engering [15] was measured in a fluid condition at 40 C., and the surface tension was 46.4 dynes. Agar of the same formula but not containing oleate had a surface tension of 56 dynes at the same temperature. It is believed in view of the data presented below, that whatever efficacy Engering's medium may have depends on its low surface tension.

Growth Exper. 1.—Two strains of B. diphtheriae and one of B. xerosis were studied. Each organism was cultivated in mediums with various quantities of oleate, glycocholate, bile, and iso-amyl alcohol. The surface tensions ranged from 56 dynes in the control, to a minimum of 30.6. Suitable controls for sterility were prepared also. Inoculation of each tube was made with one loopful of a salt suspension of the organism.

After inoculation the surface tension and P_H were measured. Some difficulty was encountered in measuring the P_H of the medium containing oleate, due to the turbidity. In no case did the P_H go beyond the optimum limits for the growth of B. diphtheriae. The tubes were incubated for 48 hours at 37 C. Measurement of growth was then made by the loop method described. By using oleate, the growth of both strains of B. diphtheriae was noticeably depressed when the surface tension was lowered to a point near 50 dynes. Below 37 dynes no proliferation took place. By using iso-amyl alcohol, growth of this organism was distinctly depressed at about 46 dynes, and inhibited entirely at about 34 dynes.

B. xerosis, however, grew luxuriantly at 49 dynes, a tension that almost completely inhibited B. diphtheriae. In iso-amyl alcohol solutions, B. xerosis flourished where B. diphtheriae failed to grow (40 dynes). The depression point (36 dynes) of B. xerosis was far below that of B. diphtheriae (50 dynes), and the inhibition point was also distinctly lower (5 dynes). The latter difference is even more marked (7 dynes) when iso-amyl alcohol is the reducent.

Glycocholate and bile seemed to have some inhibitory action not dependent solely on their lowering the air-fluid surface tension of the culture medium.

By using these reducents, sufficiently low surface tension could not be obtained to inhibit growth entirely. These substances, however, prevent good growth in a lower concentration, and at a much higher surface tension than does oleate, and therefore may be considered as more toxic than oleate. However, it has been pointed out that substances

like glycocholate and bile may exert some tension effects at the cell-fluid interface which are not manifest in measurements at the air-fluid interface.

If we accept oleate as the most bland and standard reducent, then we may fix the depression point of B. diphtheriae at 50 dynes and the complete inhibition point at 37 dynes, while B. xerosis is depressed only by surface tension as low as 36 dynes and is completely inhibited only below 32 dynes.

These results were checked by using 4 other strains of B. diphtheriae, 2 other strains of B. xerosis and 1 strain of B. hofmanni. A test showed one of the new strains of B. diphtheriae to be avirulent. The results, in major details, were exactly like the foregoing. Oleate was the only reducent used, because it is apparently the most bland substance with a powerful effect on surface tension. Here again the surface tension at which growth of diphtheria bacilli is inhibited entirely was between 35-38 dynes, depending on the strain, while that at which the xerosis bacillus fails to grow was between 32 and 33 dynes. The depression points of the organisms were clearly far apart, ranging around 50 for B. diphtheriae, and below 38 for B. xerosis and B. hofmanni. No striking difference between B. xerosis and B. hofmanni was brought out. It is suggested that these differences in reaction to low surface tension may furnish a useful means of differentiating between diphtheria and pseudodiphtheria bacilli.

The following medium is suggested for differentiating true diphtheria bacilli from diphtheroids: beef serum, 100 c c.; distilled water, 700 c c. Divide in two portions of 400 c c. each, add to one 64 c c. of 1% aqueous solution of C.P. sodium oleate and to the other 64 c c. of distilled water.

The mixtures are sterilized by the fractional method. They should not coagulate during the heating. If they do, more water is needed. The surface tension of the oleate medium should not be higher than 40 dynes nor lower than 36 dynes. The optimum is 40 dynes. The tension may be adjusted by adding more oleate or serum water as may be required.

The organism to be tested is isolated in pure culture by one of the usual methods. One tube, containing 5-10 c c. of the oleate medium and one tube of the plain serum water are inoculated and incubated for 48 hours at 37 C. Both tubes are then shaken for a minute or two to distribute the growth, and equal quantities (1.01 c c. or a standard loopful) of each are spread evenly over 5 mm. squares. The films are dried, fixed, stained with Lœffler's methylene blue, and examined under oil. If luxuriant growth occurs in both tubes, the organism may be regarded as a diphtheroid; if luxuriant growth occurs in the plain medium, but little or no growth is seen in the oleate medium, then the organism in all probability is B. diphtheriae. A known culture of B. diphtheriae may be used for comparison at first.

In discussing the experiments with the coli-typhoid group, the possibility was mentioned that similarity in reaction to changes in surface tension may be due to a likeness in colloidal and membrane structure of the organisms in question. The differences and likenesses noted in the reaction of members of the group of Corynebacteria, on the basis of similar considerations, also may be taken to denote lines of natural grouping or segregation.

It may be that the apparent inability of certain organisms to multiply at low surface tension represents simply a greater resistance on their part to a possible constrictive action of the fluid medium surrounding them. It is suggested that this constrictive action may aid in pinching the daughter cell off from the parent. The suggested nature of this pinching action, if indeed it exists, may be made clearer by the following explanation: We may consider a sausage with an elastic band around it. If we strengthen the band, it will tend to constrict on the sausage and cut off one part from the other. The force of the elastic band would be in opposition to the strength of the skin of the sausage, the nature of the contents, etc. If the elastic band should be weakened, we should have no constriction, and consequently no increase in the number of sausages. It is suggested that surface tension in the divisionof bacteria may play a rôle analogous to that of the elastic band. Evidence in support of this idea will be presented later. It is pointed out, however, that there must be, in the case of the bacteria, some sort of weakening at the middle of the cell, at the circumference and in a plane at 90 degrees to the plane of division. It is obvious that the surface tension, being exerted equally on all sides could hardly constrict more in one place than in another unless the resistance of the cell were lessened at this point by some other factor.

RECAPITULATION

Members of the Corynebacteria have been cultivated at various surface tensions. It has been found that B. diphtheriae is fairly sensitive to low surface tension, being unable to grow well in medium having a tension less than about 50 dynes. B. hofmanni and B. xerosis, on the contrary, have been found to be resistant to low surface tension. A method for differentiation between true diphtheria and pseudodiphtheria bacilli is described, which is based on differences in reaction to low surface tension.

THE PNEUMOCOCCI AND STREPTOCOCCI

Pneumococci.—The first experiments were planned to measure the ability of pneumococci to grow at low surface tensions and to study

the effect of reducents on their pathogenicity. It was necessary first to make certain modifications in the usual method of preparing mediums for these bacteria.

Medium for Cultivation of Pneumococci.—Huntoon's infusion broth was the medium in routine use. The article containing the formula for this medium[41] states that filtration through paper or cotton, or any substance having vegetable fibers with adsorbent properties, reduces the nutrient properties of the broth. In preparing broth according to Huntoon's original formula, a fine brass wire mesh had been used to strain off the larger meat particles. The clear supernatant fluid may be siphoned off after letting the material stand over night in tall cylinders. This yields an excellent product. The surface tension of this broth, however, being only 53-55 dynes, allows only a slight margin above the supposed suppression point of the pneumococcus (50 dynes). The use of charcoal was suggested to me by W. P. Larson as a means of raising the surface tension of the medium and allowing a greater range for experimental adjustment of this factor.

To a liter of Huntoon's broth, 20 to 30 gm. of bone charcoal are added, and the mixture shaken for some seconds in a flask. The charcoal is allowed to settle for 20 minutes and the material is filtered through paper. The clear filtrate is slightly greenish, and it has a surface tension, usually near 60 dynes. If the filtrate be treated with fresh charcoal, a further rise in surface tension will be obtained, although not as great as at first. The second treatment is not recommended.

The action of the charcoal may depend on surface tension reducents present in the medium collecting upon the extensive surface presented by the finely divided charcoal, and then being removed by filtration. This is possibly not the whole explanation, however, for similar treatment with kaolin was much less effective, raising the surface tension only 1 or 2 dynes as against 6 or 7 by the charcoal. It may be that this is a case of selective adsorption in which the substances in the broth have greater affinity for charcoal than for kaolin. Again, the charcoal particles may be porous and thus present a much greater surface than the solid particles of kaolin.

On repeated trial it was found, by comparing the growth in the treated and untreated broths, that the filtrate obtained as outlined was a better medium for pneumococci than the ordinary broth. In view of the data to be presented it seems reasonable to assume, for the present, that the rise in surface tension is the important, if not the only, factor in this enhancement of growth.

Experiments with Pneumococci.—The reducents selected for the growth experiments in charcoal treated broth were 1% solutions of sodium oleate, glycocholate, and pure iso-amyl alcohol. The inoculums were 0.1 c c. of the

[41] Jour. Infect. Dis., 1918, 23, p. 169.

24-hour broth cultures. The change in P_H produced in 5 c c. of the broth by addition of maximal quantities of any of the reducents was found to be negligible. The results appear in table 6.

TABLE 6

LOWEST SURFACE TENSIONS AT WHICH THE GROWTH OF PNEUMOCOCCI WAS OBSERVED TO OCCUR IN BROTH, THE SURFACE TENSION OF WHICH HAD BEEN LOWERED BY VARIOUS REAGENTS

Types	Oleate	Glycocholate	Iso-Amyl Alcohol
Type 1	53 -51 dynes	51-47 dynes	51.7-49.7 dynes
Type 2	53.1-51 dynes	51-47 dynes	51.7-49.6 dynes
Type 3	53.7-51 dynes	51-47 dynes	51.6-49.6 dynes
Group 4	53.1-51 dynes	.....	.....

The inhibition point of the 3 main serologic types is thus placed near 52 dynes. This agrees well with Larson's results. No significant difference is found between the 3 homogeneous types and this particular strain of group 4. The surface tension at which growth was visibly depressed was found to be about 53 dynes for each of the serologic types.

Studies on Pathogenicity.—One strain of type 2 was used as representative of the group. The peritoneum of a mouse dead of pneumococcus infection was washed, and the washings divided into 2 portions of 0.5 c c. each. (A blood-agar plate made at this time showed only pneumococcus colonies after 24 hours at 37 C.) To one part was added 0.5 c c. of sterile 0.85% NaCl solution, and to the other portion was added 0.5 c c. of 1% oleate solution. These mixtures were allowed to stand at room temperature for about 1½ hours, when stained preparations were made from each mixture by Gram's method and by Hiss' method for capsules. Mixture 1 showed the pneumococci in enormous numbers and pure culture with typical capsules, while no organisms were observed in mixture 2. Mice then received injections with 0.25 c c. of the mixtures. In less than 24 hours the mouse inoculated with mixture 1 (no oleate) was dead. Examinations showed the mouse to have died of a typical type 2 pneumococcus infection. The other mouse (oleate) remained as well as ever. Organisms from the heart blood of the dead mouse grew in pure culture in plain Huntoon's broth.

This result of course confirms the results of numerous other workers which showed that the pneumococcus is sensitive to sodium oleate. The work of Welch,[23] and Klotz,[24] already referred to, is of interest in this connection. The question remains, however, whether this result is due to the physical or the chemical nature of the oleate. In an attempt to answer this question a number of chemically different substances, all surface tension reducents, were selected for treatment of the pneumococcus as follows:

1. 1% oleate (soap), selected as a control on the previous experiment, 30.6 dynes.

2. 20% peptone (protein decomp. products), selected because of its wide use in culture mediums and its peculiar chemical composition, 49.1 dynes.

3. Albolene (saturated hydrocarbon), selected because of its chemical inertness and its oily character, 36.2 dynes).

4. 1% glycocholate (salt of complex organic acid), selected because of its supposed rôle in the lytic action of bile, 39.6 dynes.

5. Ethyl alcohol (95%), 27.6 dynes.

6. Bile, selected, not as a chemical entity, but because of its use in solubility experiments and in order to compare it with a glycocholate, 49.8 dynes.

7. Normal salt solution, inert control substance, 67 dynes.

Peritoneal washings from a mouse which had just succumbed to pneumococcus injection were divided into 7 portions, each of which contained 0.25 c c. of the washings. To each of these 7 parts were added, respectively, 0.25 c c. of one of the solutions listed above. After shaking and allowing the mixtures to stand at room temperature for 15 minutes, mice received injections with 0.25 c c. of each mixture. Sterile control injections were made with each reducent, substituting normal salt solution for the peritoneal washings. The conditions of the mice 18 hours after the injections are shown in table 7.

TABLE 7

SURVIVAL OF MICE AFTER INJECTION WITH TREATED PNEUMOCOCCI IN PERITONEAL WASHINGS

Mice	Mixtures Injected	Condition of Mice after 18 Hours
1	Washings + oleate	Alive and vigorous
2	.85% NaCl + oleate	Alive and vigorous
3	Washings + peptone	Alive and vigorous
4	NaCl + peptone	Dead, necropsy showed that needle had punctured liver
5	Washings + albolene	Alive and vigorous
6	NaCl + albolene	Alive and vigorous
7	Washings + glycocholate	Dead (not pneumococcus infection)
8	NaCl + glycocholate	Dead
9	Washings + 95% ethyl alcohol	Dead
10	NaCl + 95% ethyl alcohol	Dead
11	Washings + bile	Alive and vigorous
12	NaCl + bile	Alive and vigorous
13	Washings + 0.85% NaCl	Dead (after 15 hours)

It seems that the protective property, first observed in oleate, is common to surface tension reducents in general, and is not a chemical property but a truly physical one.

Mixtures similar to those injected into mice were prepared and warmed at 37 C. for various lengths of time. Microscopic observation showed that in the presence of surface tension reducents, pneumococci rapidly undergo disintegration, starting with a loss of ability to retain Gram's stain, followed by a swelling and granulation, a loss of capsule and ending by complete solution. The relation of these results to the observations by Welch seem to be of special interest.

The fact that autoclaved bile was used would appear to preclude the idea that its lytic power is due to enzyme action. It is possible that this property of bile and of surface tension reducents in general is due to some change which these substances are able to produce in the cell membrane of pneumococci.

If the tension of this membrane were lowered by surface tension reducents, the mass of protoplasm might tend to break up into a number of smaller granules and so, finally, into invisible colloidal particles. In this case the protoplasm would be dispersed by a mechanism similar to that involved in emulsification.

An additive factor may be the weakening of the retaining pressure that depends on the surface tension of the fluid surrounding the cell. Any expansive property of the cell would cause it to burst, much as a soap bubble will burst when introduced into a chamber of low gas tension.

Streptococci.—In arranging to study the effects of surface tension on streptococci, it was believed that a differentiation on the basis of reaction to low surface tension might be possible. If differences could be shown to exist only between distantly related groups, the data obtained would still have a value as being indicative of what might later be expected of this method. A fine discrimination between closely related types of streptococci was not attempted in this study. The following strains were used: (1) Streptococcus pyogenes, Hygienic Laboratory, Washington, D. C.; (2) β type (Smith and Brown [42]), sore throat; (3) β type, raw milk; (4) β type, raw milk; (5) α type (Smith and Brown [42]), normal throat; (6) γ type (Brown [43]) feces. A detailed description of the technical arrangements of these experiments is not given. They are, in fact, exactly like those used in experiments with pneumococci.

It was found that, as compared with oleate, the other reducents inhibited growth irregularly, at surface tensions too high to explain their effects solely as the result of lowered tension. To explain their action, it might be necessary to take into account the possibility that they have penetrated the cell membrane, or in some other way acted differently from sodium oleate and iso-amyl alcohol. These reducents must, therefore, have exerted on the organisms some influence not comparable with that of sodium oleate. For this reason their use in these experiments was discontinued.

[42] Jour. Med. Res., 1915, 31, p. 455.
[43] Monographs of the Rockefeller Inst. for Medical Research, No. 9, 1919.

The experiment was repeated with sodium oleate only, and the quantities more carefully graded. It may be well to note here that we are considering the results obtained with sodium oleate as a sort of comparison standard. It is, of course, realized that this consideration is based on rather meager evidence. We do not know whether oleate does or does not penetrate the cell membranes. Neither do we know these things regarding the other reagents used. But we do observe, in the data already obtained, that the effects of oleate vary much more constantly, in proportion to its reduction of surface tension, than those of other substances, and that it is much less bactericidal as a rule. In addition, oleate has an advantage, as a standard, in being obtainable practically pure, and of constant composition. It has been used by other workers and therefore puts all the data on a more easily comparable basis.

Due to an accident, strain 2 (from sore throat) was lost, so that it could not be used in this comparison. The results of the experiment were similar to those of the first one. In contrast with the uniform results obtained with B. diphtheriae and pneumococci, the data obtained show no definite point that can be assigned as one at which the growth of streptococci is depressed noticeably. While strains 1, 3 and 4 were inhibited at almost exactly the same surface tension (34 dynes) in both experiments, strains 5 and 6 were entirely unaffected and grew as well at 34 dynes as in the control.

Whether the streptococci can be divided into two groups—the indifferent species and those inhibited by low surface tension—remains to be determined by experiment with a large number of species. It may be that these wide differences between species of streptococci in resistance to low surface tension later can be correlated with other characters.

Comparison with Pneumococci.—These experiments show that an interesting difference exists between the streptococci and pneumococci. It is generally taught that these two types of organisms are more or less closely related biologically.

The data obtained show that pneumococci are sensitive to surface tensions as high as 53 dynes, while streptococci are not affected by this surface tension. The means of differentiation offered by this unlikeness should prove of practical value, in certain cases at least, where other means are not available.

Method for Differentiating True Pneumococci from Pneumococcus-Like Streptococci.—The following medium is suggested as a means of differentiation

between pneumococci and streptococci, particularly those streptococci which form short chains and are elongated and which produce the alpha type of hemolysis of Smith and Brown on blood-agar plates:

A basic medium is prepared as follows: ground lean beef, 500 gm.; water (distilled), 1,000 gm.; peptone, 10 gm.; NaCl, 5 gm.; egg (whole), 1.

The ingredients are mixed in an agate-ware vessel and heated, with constant stirring, to 68 C., or until the red color changes to brown. Coagulation should not take place at this time. Sufficient N/1 NaOH is now added to make the reaction slightly alkaline to litmus. The vessel is then put in the Arnold sterilizer for one hour when the meat and egg will have formed a fairly solid clot. The clear fluid may be poured off into another vessel, straining out the larger meat particles with a piece of fine wire gauze.

The medium is transferred to a 1,000 c c. cylinder and allowed to stand in the icebox over night. The fat collects on top and a precipitate settles to the bottom. Next day the clear fluid is siphoned off and the charcoal treatment proceeded with as follows:

One thousand c c. of infusion broth, prepared according to the modified method described, are divided into two portions of 500 c c. each and to one portion are added 35 c c. of 1% aqueous solution of sodium oleate; to the other portion 35 c c. of distilled water. The mixtures are tubed and sterilized by the fractional method. The oleate mixture should have a surface tension between 48 and 52 dynes. The tension may be adjusted by adding more broth or oleate as the case may be. The P_H should not need adjustment and should be near 7.

In making the differentiation the same procedure is followed as is prescribed for the differentiation of diphtheria from pseudodiphtheria bacilli. If luxuriant growth occurs in both the plain and oleate medium the organism may be regarded as a streptococcus, while if luxuriant growth occurs in the plain medium and little or no growth in the oleate medium, the organism is probably a pneumococcus.

The method, in addition to a practical value, may have a scientific interest. It has been suggested that surface tension reagents may affect the cell membranes. If this should be true, the data suggest that pneumococci and streptococci, however much alike they may appear in other respects, may be unlike in the structure of their cell membranes.

SUMMARY

Surface tension is an important factor in bacterial growth, morphology, and probably in cell division, and to a less extent in the motility of bacteria, and their ability to retain Gram's stain.

Differences in ability to grow at low surface tensions offer a valuable and simple means of differentiation between species of bacteria. It is possible, by measuring the ability of organisms to grow at low surface tensions to distinguish B. aerogenes from B. typhosus and B. morgani, B. diphtheriae from B. xerosis and B. hofmanni, certain resistant types of streptococci from sensitive types, pneumococci from streptococci.

The serologic types of pneumococci do not differ from one another in their sensitiveness to low surface tensions.

Low surface tension of the culture medium may be an important factor in determining the size of the individual cells, the rate and extent of cell division, and the cell grouping.

The growth-producing properties of Huntoon's "hormone" medium for pneumococci may be improved by raising the surface tension of the medium with charcoal.

When preparing medium for pneumococci, the surface tension should be raised to some point above 54 dynes.

The loss of virulence of pneumococci after treatment with surface tension reducents may be due to the fact that these bacteria disintegrate when they are subjected to low surface tension, and the dissolution of pneumococci by bile may be due to the low surface tension induced by this substance.

Sodium oleate was found to be the most useful reagent for reducing surface tension of culture mediums.

CARBON DIOXIDE AS A FACTOR IN THE GROWTH OF THE TUBERCLE BACILLUS AND OF OTHER ACIDFAST ORGANISMS

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In comparison to the fundamental nature and importance of the problem, the question of carbon dioxide utilization by bacteria has received but scanty consideration by most investigators. Wherry and Ervin [1] were the first to point out the necessity of carbon dioxide in cultivating the tubercle bacillus. Theobald Smith [2] has reported the favorable action of the gas in hastening the growth of B. abortus. Its importance as an essential factor in the cultivation of anaerobes and aerobes alike has been emphasized by Rockwell.[3]

All of the work mentioned above has been variously challenged, notably by Novy [4] and his associates, whose recent work on microbic respiration has led them to the conclusion that neither the tubercle bacillus nor the other organisms studied by them utilize carbon dioxide, and that its absence or presence in small amounts has no effect on growth. While it has been generally conceded that the growth of bacteria cultivated over alkaline solutions in closed spaces is greatly inhibited, the opponents of the carbon dioxide theory have explained this effect as being due to a dehydration of the nutrient medium by the alkali, rather than to the removal of carbon dioxide. It has therefore seemed wise to collect some quantitative data bearing on this point, and it was to this end that the following experiments were devised.

We wish first to point out some necessary considerations in the methods of attacking this problem. In case it is impossible to demonstrate any effect of the absence of carbon dioxide on the growth of an organism by the ordinary methods, two alternatives remain for further experimentation. Either the efficiency of the carbon dioxide removal must be increased, or the production of the gas by the bacteria themselves

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[1] Jour. Infect. Dis., 1918, 22, p. 194.
[2] Jour. Exper. Med., 1924, 40, p. 219.
[3] Jour. Infect. Dis., 1921, 28, p. 249; 1923, 32, p. 98; 1924, 35, p. 581.
[4] Ibid., 1925, 36, pp. 109, 168, 245, 343.

must be cut down. The latter alternative is the simplest experimentally, and may be realized by adherence to four conditions:

1. The medium used must contain no carbohydrates or other fermentable substances, such as glycerol. If the growth is not possible without their presence, their amount must be reduced to the minimum which will sustain growth. The large amounts of carbon dioxide produced from these materials is strikingly shown by the work of Novy, and since it is formed in such close proximity to the organisms themselves, there is no chance of removing it before the necessary amount has been taken up by the bacteria.

2. The protein content of the medium must be reduced to the minimum, since bacteria produce a certain amount of carbon dioxide from proteins.

3. The inoculum must be as uniformly and as thinly seeded over the medium as possible. The ideal condition in this respect would be obtained when the inoculum consisted of isolated organisms, uniformly distributed. For some reason, clumps or clusters of organisms seem to be able to retain the carbon dioxide which they produce from the proteins of the medium, and therefore grow even in the presence of a carbon dioxide absorbent.

4. The medium must be adjusted to a reaction sufficiently acid to make the carbon dioxide available for the organism, and more easily removed by the absorbent.

In other words, in order to obtain clear-cut results showing the effect of carbon dioxide on growth, it is necessary to use the leanest medium on which the organisms in question will grow, and to seed the medium as thinly as possible.

EXPERIMENTAL

Two methods were used in these experiments. In the first method, the bottoms of sterile, dry Petri dishes were weighed, to the nearest 5 mg., on an analytic balance, suitable precautions being taken to avoid contaminating the plates. The plates were then poured with the medium, and, when they had cooled, were inoculated evenly with a swab, after which the bottoms were weighed as before. Each bottom was then inverted over its top, into which 20 c c. of the solution used had been pipetted. The tops and bottoms were separated by pieces of wooden applicators and held together by two pieces of adhesive tape. The small standard desiccators of 4⅞″ internal diameter were used. One

plate was placed in each desiccator. The ground surfaces were well sealed with petrolatum, and the tops held securely in place by means of small vise clamps.

At the end of the incubation period, the plates were removed from the desiccators and the bottoms weighed immediately. From the weight of the agar obtained from the first two weighings and the total loss in weight, the percentage dehydration of the medium was calculated. This method, except for the weighings, is substantially the one used by Novy [4] in several of his experiments.

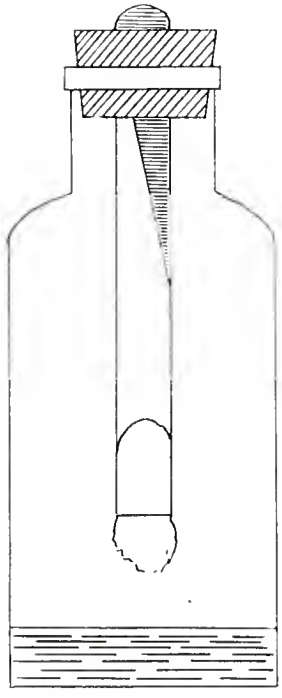

Fig. 1.—Set-up of the tube method.

The second method was designed to avoid the use of the cumbersome desiccators, and at the same time provide for a sufficient oxygen volume. Wide-mouthed bottles of 500 c c. capacity were employed, in which were placed the solutions. Tubes of slanted medium were used instead of plates. The sterile, dry tubes were first weighed empty. The medium was then added with a pipet, allowed to harden in a slanted position, and inoculated. After being weighed again, the tubes were pushed, mouths down, through the holes of tight fitting, one-holed rubber stoppers, which were placed in position in the wide-mouthed bottles as shown in fig. 1. All the joints were sealed with paraffin.

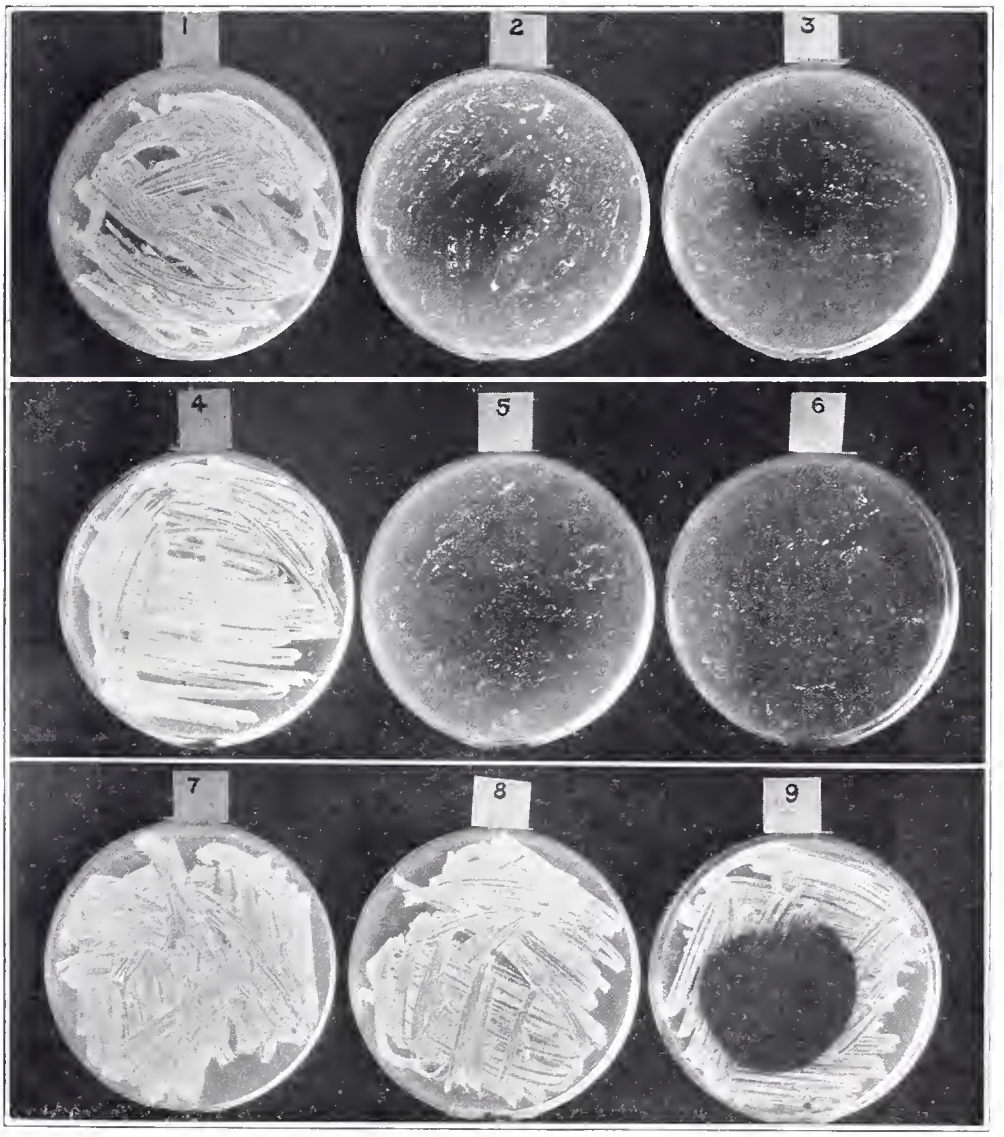

Fig. 2.—Growth of the saprophytic tubercle bacillus on meat extract agar plates incubated over the following solutions: no. 1, air control; no. 2, 2.5% KOH; no. 3, 5% KOH; no. 4, H_2O; no. 5, 10% KOH; no. 6, 20% KOH; no. 7, 10% H_2SO_4; no. 8, 20% H_2SO_4; no. 9, 40% H_2SO_4.

At the end of the experiment the tubes were removed from the stoppers, weighed, and the percentage dehydration of the medium obtained as before.

This procedure was found to be particularly well suited to the experiments with the slow growing, virulent strains. Experiments with these organisms were first attempted by the desiccator-plate method, but it was found that in spite of every precaution, mold spores from the air in the desiccators lodged on the surfaces of the plates, which in a week or two were covered with a contaminating growth. The use of the bottles and plugged tubes completely removed these difficulties. It is, in our opinion, a highly significant fact that while these contaminants were always on the plates over the acids and the water controls, not once did they appear over the alkaline solutions.

TABLE 1

Saprophytic Tubercle Bacillus Incubated 12 Hours at 37 C.

Incubated Over 20 C c.	Growth	% Dehydration of Medium
Control	++++	4.2
10% H_2SO_4	++++	4.1
20% H_2SO_4	++++	16.2
30% H_2SO_4	++++	27.1
40% H_2SO_4	++++	40.5
2% NaOH	±	2.2
5% NaOH	±	5.6
10.5% $NaHCO_3$	++++	4.3

EXPERIMENTS WITH A SAPROPHYTIC TUBERCLE BACILLUS

The first work was done with an avirulent, saprophytic tubercle bacillus, the K1 strain, which grows well in 10-12 hours on ordinary meat extract agar. This is the same strain used by Wherry [5] in his work with synthetic mediums.

In table 1 is shown the growth of this organism on plates incubated over solutions of various strengths of NaOH and H_2SO_4, together with the percentage dehydration of the medium in each case. The medium used was meat extract agar of reaction + 0.5. The solution of $NaHCO_3$ was so calcuted as to be equivalent to a 5% solution of NaOH saturated with CO_2. It is seen from table 1 that, while growth is inhibited over NaOH, it occurs over NaOH saturated with CO_2, namely, over $NaHCO_3$. It is also apparent from the figures in the right hand column that the inhibition over NaOH cannot be due to loss of moisture from the medium.

[5] Jour. Infect. Dis., 1913, 13, p. 144; Centralbl. f. Bakteriol., I. O., 1913, 70, p. 115.

Fig. 3.—Showing growth of a virulent human tubercle bacillus on Petroff's medium: tube 1, over H_2O; tube 2, over 4% NaOH; tube 3, over 20% H_2SO_4. Note the dehydration of the medium in tube 3.

Table 2 gives the results of a set in which KOH was used as the alkali. This is illustrated photographically in fig. 2.

Table 3 shows the results obtained when dehydrating agents other than H_2SO_4 were used.

These experiments are typical of many which were performed with this organism, and which gave uniformly consistent results with the same experimental procedure. More than 60 plates were incubated under the various conditions, and in every case growth was inhibited on the plates over alkaline solutions, although the mediums were less dehydrated than those incubated over acid, on which good growths were obtained.

TABLE 2

SAPROPHYTIC TUBERCLE BACILLUS INCUBATED 22 HOURS AT 35 C.

Incubated Over 20 C c.	Growth	% Dehydration of Medium
Control	+++	5.4
10% H_2SO_4	++++	5.3
20% H_2SO_4	+++	14.9
40% H_2SO_4	++	33.8
5% KOH	±	4.3
20% KOH	±	26.4

TABLE 3

SAPROPHYTIC TUBERCLE BACILLUS INCUBATED 20 HOURS AT 37 C.

Incubated Over	Growth	% Dehydration of Medium
Control	++++	4.6
30% glycerol (15 c c.)	+++	21.0
$CaCl_2$ (0.25 gm. dry)	+++	24.5
4% NaOH (20 c c.)	+	7.7

Such growths as did appear on the plates incubated over alkali were of an entirely different type from those on the other plates. The latter were always uniform, confluent growths, while the former, in every case, were of a granular character, consisting of more or less isolated clumps. This is due, as mentioned in the foregoing, to the fact that at the points where these clumps appear the inoculum consisted of masses of bacilli, which are able to survive and grow. Furthermore, if one of these plates which has been incubated over alkali be removed from the desiccator and again incubated, with a plentiful supply of air, no other growth appears on the plate; only the clumps continue to grow. This indicates that the bacilli between these clumps

have died off, evidently from lack of carbon dioxide. Novy[4] also mentions this characteristic appearance of the growth obtained over alkali.

EXPERIMENTS WITH VIRULENT STRAINS

Tables 4 and 5 give the results obtained with two virulent strains of tubercle bacilli—a bovine and a human strain.

The experiment with the bovine strain was repeated three times, with equally consistent results. Fig. 3 is a photograph of the experiment with the human strain given in table 5. The great dehydration

TABLE 4

VIRULENT BOVINE TUBERCLE BACILLUS, 3% GLYCEROL AGAR, REACTION + 1; INCUBATED 10 DAYS AT 37 C.

Incubated Over 35 C c.	Growth	% Dehydration of Medium
Control, H_2O	++	2.3 (gain)
30% glycerol	+++	9.8
50% glycerol	+++	13.7
20% H_2SO_4	+++	7.8
4% NaOH	±	0.5

TABLE 5

VIRULENT HUMAN TUBERCLE BACILLUS, PETROFF'S MEDIUM; INCUBATED 25 DAYS AT 37 C.

Incubated Over 35 C c.	Growth	% Dehydration of Medium
H_2O	+++	1.9 (gain)
20% H_2SO_4	++++	18.1
4% NaOH	±	0.8 (gain)

of the culture over acid is evident, the medium having dried away from the sides of the tube. The tube over alkali actually gained slightly in weight. This was frequently noticed in the experiments conducted over a long time, and is probably caused by the fact that the weak alkali was used up by absorption of carbon dioxide and by attack on the glass, leaving eventually only a solution of carbonate with silicates in suspension. The richer growth over acid than on the water control, a phenomenon which was encountered in all of our work, is no doubt to be attributed to the effect of the acid tension. The experiments with glycerol and calcium chloride, however, rule out any possibility of explaining the observed differences in growth on this basis.

EXPERIMENTS WITH OTHER ACID-FAST ORGANISMS

These experiments have been extended to two other acid-fast organisms, namely, Müller's mist bacillus and a grass bacillus. The results of these experiments are given in tables 6 and 7, respectively.

TABLE 6

Müller's Mist Bacillus, Meat Extract Agar, Reaction + 0.5; Incubated 17 Hours at 37 C.

Incubated Over 20 C c.	Growth	% Dehydration of Medium
H_2O	++	4.4
10% H_2SO_4	++	5.0
20% H_2SO_4	+++	10.2
5% KOH	+	3.3

TABLE 7

Grass Bacillus, 9.5 c c. Meat Infusion Agar (Reaction + 1), 0.5 c c. Ascitic Fluid; Incubated 18 Hours at 37 C.

Incubated Over 20 C c.	Growth	% Dehydration of Medium
H_2O	++++	2.4
10% H_2SO_4	++++	14.8
20% H_2SO_4	++++	37.7
4% NaOH	±	3.9

SUMMARY

It is shown that the inhibition of growth of a saprophytic tubercle bacillus, 2 strains of virulent tubercle bacilli, and 2 other acid-fast organisms, when incubated over alkalis in closed spaces, cannot be explained as being due to dehydration of the nutrient medium, since growth occurs over other equally or more efficient dehydrating agents which are not carbon dioxide absorbents, such as sulphuric acid, calcium chloride and glycerol. The only explanation which can account for this phenomenon is that carbon dioxide is, in some way, a vital factor in growth.

VIABILITY AND TOXICOGENIC POWER OF SMALL NUMBERS OF SPORES OF CL. BOTULINUM SUBJECTED TO DIFFERENT TEMPERATURES *

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Many investigators have studied carefully the problem of the resistance of the spores of Cl. botulinum to heat. The relationship of these spores to heat has been satisfactorily determined when the organisms have been cultivated in the ordinary mediums in which the physical and chemical conditions could be under reasonable control, as well as in the varied and more complex natural mediums, such as vegetables, fruits, etc. These experiments have always been carried out using comparatively large numbers of spores as the inoculum. Since it is a common observation that only a few cans, at most, of any pack of canned vegetables undergo botulinus spoilage, though presumably all are subjected to the same processes, study of the factors involved in such contamination was thought advisable as a means of throwing light on this condition.

This series of experiments was designed to discover whether there is any relationship between the number of spores introduced into a given quantity of medium and the sterilizing power of heat. Experiments dealing with the thermostability of the spores of Cl. botulinum when present in large numbers have been carefully carried out and reported by Esty and Meyer.[1] No record of the resistance of small numbers of spores is found in the literature. In our experiments, small numbers of spores, used as the inoculum, were introduced into various mediums, and these were then subjected to different temperatures. After varying periods of incubation—at room temperature, at 25 C., and at 37 C.—they were observed for evidence of growth and tested for toxin production.

TECHNIC

Medium.—Eight different types of medium were employed in this work, viz., sweet corn having a P_H of 7 and 6.2; peas, P_H 7.6 and 5.9; spinach, P_H 7.6

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[1] Jour. Infect. Dis., 1922, 31, p. 650.

and 5.3; string beans, P_H 7.6; beef heart medium, P_H 7.8, this last being used as a control.

The different reactions were (1) those found naturally in the canned material, and (2) those resulting from adjustment with Na_2CO_3 following laboratory titration. No change could be observed in the physical appearance of any of the mediums when rendered more alkaline, except in the case of the spinach, in which the adjustment of the reaction to a P_H of 7.6 resulted after sterilization in a product much darker and not so firm.

In the case of the vegetables, 25 gm. of the medium were placed in large pyrex tubes and sterilized under a pressure of 15 pounds for 1 hour. The tubes were then incubated for 1 week before being used in order to be assured of adequate sterilization.

Cultures.—A and B types of Cl. botulinum were used in these tests. Both cultures were descended from single cell isolations, and had been checked repeatedly for purity and toxicogenic powers. The A type was our $M7a_2$ culture first isolated from olives responsible for the Greensburg outbreak of botulism. The B type was our 126.7 culture, isolated originally from corn ensilage by Dr. Robert Graham of the University of Illinois. These organisms had been grown in pea juice for 2 months at 37 C. Examination of the cultures showed highly refractive spores, with only an occasional vegetative cell. Inoculations of agar slants proved the purity of the cultures as regards the presence of aerobic contaminants, while subcultures of massive inoculations into beef heart medium yielded abundant typical growths with the production of the usual potent toxin. The spores were removed by centrifuging, washed three times with sterile salt solution, and resuspended in a salt solution. They were detoxified by the addition of homologous antitoxin. Tested by injections into white mice, these spore suspensions proved nontoxic.

Inoculations.—A suitable dilution of the stock suspension was made and the desired number of spores to be used as the inoculum were then picked out by means of the Barber technic. The selected spores, after being discharged into a separate small drop on the cover glass, were taken up with another sterile capillary pipet and transferred to the appropriate medium. The time of exposure of such spores to oxygen, light and dryness was not accurately measured, but the average time of isolation was from 4 to 5 minutes.

Three abnormal temperatures were used to modify the inoculated spores; exposure to 70 C. for 10 minutes, to 100 C. for 10 minutes, and to 120 C. for ten minutes. One series of each medium was kept unheated to serve as control. In the case of the 70 C. and 100 C. exposures, the mediums were at the desired temperatures when the inoculations were made and after 10 minutes at such temperature, the tubes were cooled by immersion in cold water. In the case of the exposure to 120 C., the time is only an approximation, the heating being carried out in the autoclave. The medium was heated to 100 C. and the inoculation made; in the meantime, the autoclave had been heating, and the tubes were introduced immediately after inoculation. One minute was allowed for the desired temperature to be reached, and they were kept at that temperature for nine minutes, allowing for 1 minute to reduce the pressure. On removal they were cooled quickly by immersion in cold water. Anaerobiosis was established by sealing the tubes with sterile petrolatum.

Six inoculations of each organism were made into each of the 8 types of medium for each of the 4 exposure temperatures, i. e., unheated, 70 C. for 10 minutes, 100 C. for 10 minutes, and 120 C. for 10 minutes. This made a total of 192 cultures for each organism used, or a total of 384 cultures for the

A and B type organisms studied. Two tubes of each inoculation were incubated at room temperature, 2 at 27 C. and 2 at 37 C.

Observations of the inoculated material for evidence of growth and toxin production were made at weekly intervals over a period of 124 days. The viability of the organisms and the purity of culture were determined by direct observations and by subculturing in beef heart medium and on veal agar slopes.

The toxin production was determined by the removal of small quantities of the material at the time of observations for use in making intraperitoneal injections into white mice. No attempt was made to measure quantitatively the amount of toxin produced. All injections into mice consisted of 0.25 c c. of a 1:50 dilution of centrifuged medium.

Table 1 gives the details regarding the test with 126.7 (type B). The results obtained with $M7a_2$ (type A) were identical in every detail, and hence need not be tabulated nor described.

The results were uniform throughout. That the exposure of the original spores to the light and oxygen necessarily attendant on isolation did not reduce the viability, is evident from the fact that in no instance did the tubes inoculated with unheated cells fail to show growth in about the usual length of time and with normal toxin production.

Exposure to temperature of 70 C. for 10 minutes did not destroy the spores, and in every instance growth took place after such exposure, although there were indications of retarded development. Without exception, no growths were obtained in any tubes in which the isolated spores had been subjected to a temperature of 100 C. or 120 C. for 10 minutes—not even after an incubation at 37 C. of 124 days. Subcultures into beef heart medium made from these negative tubes invariably resulted in the absence of growth. Thus it would seem that the number of spores of Cl. botulinum entering into the contamination of vegetables plays some part in the resistance of the organism to heat, and may indicate one factor responsible for the appearance of botulinus in certain cans in a pack but not in others. The uneven distribution of the spores may explain heavier infection in certain cans than in others, with corresponding differences in their resistance to the heat applied in the process of canning.

No differences of any significance were observed in the cultures incubated at room temperature, at 27 C. and at 37 C. In general, growths appeared somewhat earlier at the higher temperatures, although this was by no means a constant difference.

CONCLUSIONS

The thermal death point of limited numbers of spores of Cl. botulinum inoculated into ordinary vegetables such as peas, corn, string beans,

TABLE 1

VIABILITY AND TOXICOGENIC POWER OF SPORES SUBJECTED TO DIFFERENT TEMPERATURES

Peas, PH 7.6; inoculum 9—12 spores of culture 126.7

Temperature to Which Spores Were Subjected	Results: Incubation Temperatures — Room Temperature				27 C.				37 C.			
	Growth		Toxin Production		Growth		Toxin Production		Growth		Toxin Production	
Unheated	+++	10	+++	12	++++	12	++++	12	++++	12	++++	12
70 C. 10 m.	+++	10	+++	12	++++	12	++++	12	++++	12	++++	12
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
Peas, PH 5.9; inoculum 9—12 spores of culture 126.7												
Unheated	+	13	++	13	+++	13	+++	13	++	13	+++	13
70 C. 10 m.	++	37	+++	30	+++	30	+++	30	+++	30	+++	30
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
Corn PH 7; inoculum 9—12 spores of culture 126.7												
Unheated	+++	27	+++	27	++++	27	++++	27	++++	8	++++	10
70 C. 10 m.	+++	27	+++	27	++++	27	++++	27	++++	8	++++	10
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
Corn. PH 6.2; inoculum 9—12 spores of culture 126.7												
Unheated	++	27	+	27	+	27	+	27	++++	27	++++	27
70 C. 10 m.	+	27	+	27	+	27	+	27	+	27	+	27
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
Spinach. PH 7.6; inoculum 9—12 spores of culture 126.7												
Unheated	+	27	+	27	+++	20	+++	20	++++	10	++++	10
70 C. 10 m.	+	54	+	54	+++	27	+++	27	+++	18	+++	18
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
Spinach, PH 5.3; inoculum 9—12 spores of culture 126.7												
Unheated	++++	54	+++	54	++	20	++	20	±	54	0	54
70 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
String beans. PH 7.6; inoculum 9—12 spores of culture 126.7												
Unheated	+++	27	+++	27	++++	20	++++	20	++++	20	++++	20
70 C. 10 m.	+++	27	+++	27	++++	27	++++	20	++++	20	++++	20
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
Beef heart medium, PH 7.8; inoculum 9—12 spores of culture 126.7												
Unheated	+++	20	+++	20	++++	20	++++	20	++++	10	++++	10
70 C. 10 m.	+++	20	+++	20	++++	20	++++	20	++++	10	++++	10
100 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124
120 C. 10 m.	0	124	0	124	0	124	0	124	0	124	0	124

The figures under growth and toxin production refer to days.

and spinach, is much lower than when mass contamination occurs. In a series of 384 cultures, with an inoculum of 9 to 12 detoxified spores in each culture, no growths were obtained subsequent to heating the inoculated tube at 100 C. for 10 minutes. Growths were uniformly obtained when similar cultures were heated at the lower temperature of 70 C. for the same length of time.

Within these limits of exposure temperatures, the thermal death point is independent of the incubation temperature, practically the same results being obtained when the inoculations were kept at room temperature, at 27 C., or at 37 C.

No significant differences were observed in the results that might be dependent on the different varieties of vegetables used, except in the case of the spinach, in which growths were less rapid even when its reaction had been adjusted to a P_H of 7.6. When the spinach in a natural state, with a P_H of 5.3, was inoculated, growths and toxin production were scanty even in the unheated cultures, and none appeared in these cultures when the spores had been heated at 70 C., or a higher temperature, for 10 minutes.

RELATIONSHIP OF INCUBATION TEMPERATURE TO VIABILITY, RATE OF GROWTH AND TOXIN PRODUCTION OF CL. BOTULINUM IN DIFFERENT VEGETABLES *

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A review of the literature on Cl. botulinum reveals a marked lack of uniformity of opinion regarding the relationship of temperature to the metabolism of the organism. Early workers denied the ability of the organism to grow and produce toxin at the temperature of the body, and established their optimum for these functions at 20 to 25 C. Such findings are so commonly cited in the earlier reviews on the subject that it is not necessary to cite them individually at this time.[1] The more recent work in this country with the strains of Cl. botulinum isolated by American workers has proved the fallacy of this view, and has established the fact that this organism is capable of both growth and toxin production at 37 C. This discrepancy in results is difficult of explanation except on the basis of a difference in the thermal characteristics of the various strains.

The ability of the organism to grow and to produce toxin in a vegetable medium, as well as in meat, was first reported by Landmann [2] in connection with his investigation of the outbreak of botulism at Darmstadt. Since that time, the demonstration of botulinus toxin in various fruits and vegetables has been reported frequently. A complete review of the literature dealing with this phase of the study may be found in the articles of Dubovsky and Meyer,[3] and Geiger, Dickson, and Meyer.[4]

The purpose of the series of experiments herewith reported was to gain some information relative to the rate of growth, toxin production, etc., in different vegetables at different temperatures of incubation.

Two strains of Cl. botulinum—$M7a_2$ and $276B_7$—both cultures resulting from single cell isolations, were used in this work. That they

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[1] Van Ermengem: Handb. der Path. Mikroorg., 1912, 4, p. 909. Dickson: Monograph Rockefeller Inst. Med. Res., 1918, No. 8.

[2] Hyg. Rundschau, 1904, 14, p. 449.

[3] Jour. Infect. Dis., 1922, 31, p. 501.

[4] Bulletin No. 127, U. S. P. H. S., 1922.

are both highly toxicogenic strains was demonstrated in earlier work on this organism which has already been reported. $M7a_2$ is a type A organism which was responsible for the outbreak of botulism at Greensburg, Pa., and was obtained from the collection of Dr. Karl Meyer. Culture $276B_7$ is a type B organism. The original stock from which the isolation was made was received from Dr. Robert Graham of the University of Illinois.

These organisms were cultivated in casein-digest veal infusion broth having a reaction of P_H 7.6 after sterilization. They were grown in this medium for three weeks at 37 C., at the end of which time, examination revealed the presence of free spores with only an occasional vegetative cell. The cultures were centrifuged at high speed, washed twice with sterile salt solution, after which they were resuspended in the sterile saline, and the number of spores per unit volume determined by direct count, using a hemocytometer. Shake cultures in veal infusion agar were also made from the washed suspension of cells in order to obtain an approximate idea of the number of viable spores present. The inoculum consisted of 1,000,000 spores (direct count) for each tube, or can, inoculated. As nearly as could be determined from the colonies developing in the shake cultures, this represented 750,000 viable spores. Prior to the inoculation, suspensions used were heated to 80 C. for 20 minutes in order to destroy any toxin present. The efficiency of this degree of heat for the destruction of toxin was determined by intraperitoneal injections into mice. None of the animals showed any evidence of botulinus intoxication, although the quantities injected were many times the ordinary lethal dose.

MEDIUMS USED

For the purpose of studying these temperature relationships to toxin production and growth, corn, peas, green string beans, and spinach were used. These mediums were used in two ways:

(1) Inoculations were made directly into No. 2 cans. The surface of each can was sterilized; it was thoroughly washed for 15 minutes with 5% phenol; the top of the can was then washed with cotton soaked in 95% alcohol; the alcohol was ignited, and the can was rubbed with the cotton and burning alcohol. An opening was made by puncturing the top of the can with an ordinary ice pick heated to red heat. Through this opening—only large enough to admit a small Pasteur pipet—the organisms were introduced. The can was immediately resealed by soldering. The puncturing of the can for inoculation purposes evidently destroyed the reduced oxygen tension owing to the process of canning; but the outcome of the experiment is sufficient evidence that it did not interfere with anaerobic conditions to a degree which would affect materially the growth of Cl. botulinum.

(2) In the other method, 50 gm. of each medium were placed in large sterile pyrex tubes. A few cubic centimeters of distilled water were added, and the medium was then sterilized in the autoclave at 15 pounds' pressure for 45 minutes. To determine the sterility of the mediums, all cans and tubes were incubated at 37 C. for from 3 to 5 days; at the end of that time, from various tubes selected from near the centers of the baskets, transfers were made to agar slants and to freshly boiled beef heart medium under petrolatum. Subjection to 3 additional days of incubation at 37 C. followed. Prior to inoculation, all the tubes were boiled for 15 minutes to expel any absorbed oxygen; they were then cooled quickly. The inoculations having been made with Pasteur pipets, the medium in the tubes was covered with sterile petrolatum to a depth of from three quarters inch to one inch.

Inoculations were always made from cans and tubes at the time of introducing the inoculum, as a check on the sterility. Both aerobic and anaerobic methods of cultivation were employed.

The hydrogen ion concentration and the titratable acidity of each can and tube of medium were determined at the time of its inoculation. The determination of the hydrogen ion concentration was accomplished colorimetrically, using the method of Clark and Lubs. The total acidity was measured by titration with phenolphthalein, using n/50 NaOH, and is expressed in terms of the number of cubic centimeters of n/50 NaOH required to neutralize 1 c c. of the vegetable. The natural reaction of the mediums was not modified.

Three No. 2 cans and three 50 gm. tubes of each vegetable were inoculated with each organism and for each temperature to be employed. Cultures of freshly boiled beef heart medium were inoculated also with each organism for each temperature as controls.

TEMPERATURES OF INCUBATION EMPLOYED

Three different temperatures of incubation were used—10 to 12 C., 19 to 21 C., and 24 to 26 C. Cultures were not incubated at 37 C., since earlier work had shown an abundance of toxin production at that temperature. It is true also that 37 C., is not a temperature at which canned goods are ordinarily stored.

OBSERVATIONS

Subcultures and tests of toxin production were made at varying intervals of time extending over a period of 5 months. The usual procedure was to test only one can or tube for each temperature at a time, thus reserving 2 unopened until later periods. Care in opening the container and in removing material was always exercised, in order to avoid any contamination, and after each test, the container was again resealed. In this way several tests were made on each individual can or tube. When a second can or tube was opened, it was compared with the first, as regards appearance, viability of the organism and spore and toxin production. The toxicity was determined by injections into white mice, using a series of 3 dilutions—1 : 10, 1 : 50, and 1 : 250, in order to gain some idea of the quantitative production, such injections always being controlled by the use of homologous antitoxin. The viability of the spores was determined by the inoculation of the material into freshly boiled beef heart medium under petrolatum. Agar slopes were inoculated as a check on aerobic contaminants. These tubes were incubated at 37 C. for from 12 to 18 days. The agar slopes uniformly remained sterile. In those instances in which direct injections into mice of the contents of the container yielded negative toxic results, the anaerobic subcultures from that particular specimen were examined

after a suitable incubation period. If there was any evidence of growth, with gas production, a morphologic study of the contained organism was made, and injections were made into mice with some of the centrifuged broth culture to determine whether viable and toxicogenic spores were still present in the container. Proper antitoxin controls were always employed. Owing to the fact that rapidity in manipulations was desirable, no attempt was made to measure accurately the quantity of material used for making the aerobic and the anaerobic subcultures; ordinarily from 4 to 5 drops from a Pasteur pipet were poured on the agar slopes and about 10 drops in the beef heart medium. All mice used for test purposes were placed in clean, disinfected cages, where they were fed and kept under observation for at least 3 days before receiving injections.

At the time of inoculation the appearance of all the mediums was good, there was no turbidity, and the odor was normal. Mediums 1 (peas in tube) and 5 (peas in original can) were inoculated with organism $276B_7$: their P_H was 6 and the total acidity was 1.25 c c. Medium 2 (string beans in tube) was inoculated with $276B_7$ and medium 6 (string beans in original can) with $M7a_2$; their P_H was 5.6 and the total acidity was 1. c c. Medium 3 (corn in tube, with P_H 6 and total acidity 0.95 c c.) and medium 4 (spinach in tube with P_H 5.8 and total acidity 1.25 c c.) were inoculated with $M7a_2$.

Table 1 shows the results obtained when testing the various samples at the times indicated, extending over a period of 5 months. At observation 1, after a period of 72 hours' incubation, the appearance of all the mediums remained normal, hence is not indicated in the table.

An examination of the table reveals several points of interest:

1. Vegetables inoculated with detoxified spores of Cl. botulinum and incubated at low temperatures, as 10 to 12 C., showed no recognizable changes in the gross appearance, in the acidity, or in the odor. Likewise, but little toxin was produced under these conditions. Only an occasional mouse showed evidence of slight illness, and no deaths occurred among mice receiving inoculations of the mediums in dilutions of 1 : 10. That such low temperature incubation did not serve to kill the spores is evidenced by the fact that transplants of such vegetative cultures into beef heart medium, incubated at 37 C., for 18 days, resulted uniformly in the growth of Cl. botulinum and in the production of a potent toxin, just as was the case with all subcultures into beef heart medium which were made at the time of each observation from all the vegetables. The determining factor seemed to be that the low temperatures were not suitable to growth of the introduced spores. No significant changes were noted even after 5 months' incubation at such temperatures. As was to be expected, there were no macroscopic changes in the character of the mediums that were detectable by sight or by smell.

2. No marked difference can be noted between the vegetables kept at 20 C. and those at 25 C. The initial appearance of macroscopic changes

TABLE 1

OBSERVATIONS ON VEGETABLES INOCULATED WITH CL. BOTULINUM

Incubation Temperature	Medium	Observation 1 72 Hours' Incubation						Observation 2 7 Days' Incubation						Observation 3 14 Days' Incubation					
		P_H	Odor	Appearance	Toxicity for Mice Dilutions 1:10	1:50	1:250	P_H	Odor	Appearance	Toxicity for Mice Dilutions 1:10	1:50	1:250	P_H	Odor	Appearance	Toxicity for Mice Dilutions 1:10	1:50	1:250
25 C.	Peas	6.4	.....	.....	*S 12	S 12	0	6.8	Foul	Abn.	D 12	D 12	D 12	7.0	Putrid	Abn.	D 12	D 24	D 36
	String beans	5.6	.....	.....	S 12	S 12	S 12	5.0	N/1	N/1	0	0	0	5.4	Acid	N/1	S 24	S 36	S ± 36
	Corn	6.3	.....	.....	D 12	D 12	D 12	6.8	Acid	N/1	D 12	D 12	D 12	6.9	Acid	Abn.	D 12	D 12	D 12
	Spinach	5.4	.....	.....	S 12	S 12	0	5.8	Acid	N/1	S 12	0	0	5.8	N/1	N/1	S 48	S 36	S 24
	Peas	6.1	.....	.....	0	0	0	7.0	Acid	Abn.	D 36	D 36	D 72	7.0	Putrid	Abn.	D 24	D 60	S 12
	String beans	5.5	.....	.....	S 12	S 12	0	5.5	Acid	N/1	S 12	0	0	5.6	Acid	N/1	S 36	S 36	0
20 C.	Peas	6.2	.....	.....	S 24	S 12	0	7.0	Foul	Abn.	D 24	S 36	S 36	6.9	Putrid	Abn.	D 36	S 24	0
	String beans	5.4	.....	.....	S 12	S 12	0	5.4	Acid	N/1	0	0	0	5.5	Acid	N/1	0	0	0
	Corn	6.0	.....	.....	D 36	D 24	D 36	7.0	Acid	N/1	D 24	D 24	D 24	7.0	Acid	Abn.	D 12	D 12	D 24
	Spinach	5.5	.....	.....	0	0	0	5.8	N/1	N/1	S 36	S 36	S 36	5.6	N/1	N/1	S 24	S 48	0
	Peas	6.0	.....	.....	S 12	0	S 48	7.2	Putrid	Abn.	D 36	S 36	0	7.0	Putrid	Abn.	D 48	S 12	S 12
	String beans	5.6	.....	.....	S 36	0	0	5.6	N/1	N/1	S 48	S 48	0	5.4	Acid	N/1	S 24	S 12	0
10-12 C.	Peas	6.4	.....	.....	0	0	0	6.6	N/1	N/1	0	0	0	6.6	N/1	N/1	0	0	0
	String beans	5.3	.....	.....	S 36	S 36	0	5.4	N/1	N/1	0	0	0	5.4	N/1	N/1	0	0	0
	Corn	6.1	.....	.....	S 60	S 48	0	5.8	N/1	N/1	0	0	0	6.8	N/1	N/1	S 36	S 48	0
	Spinach	5.4	.....	.....	0	S 36	0	5.8	N/1	N/1	0	0	0	5.6	N/1	N/1	S 60	0	0
	Peas	6.1	.....	.....	S 60	0	0	6.4	N/1	N/1	0	0	0	6.8	N/1	N/1	S 36	S 36	0
	String beans	5.6	.....	.....	0	0	0	5.7	N/1	N/1	S 36	S 48	0	5.4	N/1	N/1	0	0	0
		Observation 4 21 Days' Incubation						Observation 5 28 Days' Incubation						Observation 6 39 Days' Incubation					
25 C.	Peas	7.0	Putrid	Abn.	D 12	D 12	D 24	6.8	Putrid	Abn.	D 12	D 12	D 24	6.8	Putrid	Abn.	D 12	D 36	D 48
	String beans	5.4	Acid	N/1	S 24	S 60	S 60	5.0	Acid	Abn.	D 12	D 12	D 24	5.6	Acid	N/1	D 24	D 36	S 48
	Corn	6.8	Acid	Abn.	D 12	D 36	D 36	6.8	Acid	Abn.	D 12	D 12	D 12	6.9	Acid	Abn.	D 12	D 12	D 12
	Spinach	5.8	N/1	N/1	S 36	S 36	0	5.8	N/1	N/1	0	0	0	5.6	Acid	N/1	S 12	S 12	0
	Peas	6.9	Putrid	Abn.	D 36	D 48	D 60	7.0	Putrid	Abn.	D 36	D 36	D 84	7.0	Putrid	Abn.	D 36	D 48	S 24
	String beans	5.6	Acid	N/1	S 24	S 36	S 36	5.5	Acid	N/1	0	0	0	5.6	Acid	N/1	0	0	0

20 C.	Peas.....	6.9	Putrid	Abn.	D 24	S 24	0	7.0	Putrid	Abn.	D 12	S 48	S 48	6.8	Putrid	Abn.	D 12	D 24	S 12
	String beans...	5.8	Acid	N/1	0	0	0	5.4	Acid	Abn.	0	0	0	5.7	Acid	Abn.	0	0	0
	Corn.....	6.8	Acid	Abn.	D 12	D 12	D 36	7.0	Acid	Abn.	D 12	D 12	D 12	6.8	Acid	Abn.	D 12	D 12	D 12
	Spinach.....	5.8	N/1	N/1	S 36	S 60	0	5.8	N/1	N/1	S 48	S 48	S 48	5.6	Acid	N/1	D 60	S 12	S 12
	Peas.....	6.8	Putrid	Abn.	D 48	S 12	S 48	7.2	Putrid	Abn.	D 36	S 48	0	6.9	Putrid	Abn.	D 24	D 60	S 12
	String beans...	5.8	Slight acid	N/1	S 24	S 36	0	5.6	N/1	N/1	S 36	S 48	0	5.6	N/1	N/1	S 48	S 24	S 12
10-12 C.	Peas.....	6.9	N/1	N/1	0	0	0	6.5	Slight acid	N/1	0	0	0	6.8	Acid	N/1	S 12	0	0
	String beans...	5.8	Slight acid	N/1	0	0	0	5.4	Slight acid	N/1	0	0	0	5.6	N/1	N/1	0	0	0
	Corn.....	6.8	Slight acid	N/1	S 48	S 48	0	6.2	N/1	N/1	0	0	0	5.9	N/1	N/1	S 12	0	0
	Spinach.....	5.8	N/1	N/1	S 60	S 60	0	5.8	N/1	N/1	0	0	0	5.4	N/1	N/1	0	0	0
	Peas.....	6.8	N/1	N/1	S 36	S 48	0	6.6	N/1	N/1	0	0	0	6.9	N/1	N/1	0	0	0
	String beans...	5.8	Slight acid	N/1	0	0	0	5.7	N/1	N/1	S 60	S 48	0	5.4	N/1	N/1	S 12	0	0
			Observation 7 46 Days' Incubation						Observation 8 2 Months' Incubation						Observation 9 5 Months' Incubation				
25 C.	Peas.....	6.6	Putrid	Abn.	D 12	D 48	D 48	6.8	Putrid	Abn.	D 12	D 24	D 36	6.8	Putrid	Abn.	D 12	D 48	D 48
	String beans...	5.6	Acid	N/1	D 36	D 36	D 60	5.8	Acid	N/1	D 36	D 36	S 12	5.7	Acid	N/1	D 36	D 36	D 48
	Corn.....	6.9	Acid	Abn.	D 12	D 12	D 12	6.8	Acid	Abn.	D 12	D 12	D 12	6.7	Acid	Abn.	D 12	D 12	D 12
	Spinach.....	5.6	Acid	N/1	S 12	0	0	5.7	Acid	N/1	S 12	S 12	0	5.6	Acid	N/1	S 12	S 12	0
	Peas.....	7.0	Putrid	Abn.	D 12	D 12	D 12	6.9	Putrid	Abn.	D 12	D 12	D 12	6.8	Putrid	Abn.	D 12	D 12	D 12
	String beans...	5.6	Acid	N/1	0	0	0	6.0	Acid	N/1	S 12	0	0	5.5	Acid	N/1	S 12	S 24	0
20 C.	Peas.....	6.8	Putrid	Abn.	D 12	D 12	S 12	6.8	Putrid	Abn.	D 12	D 36	S 36	6.8	Putrid	Abn.	D 12	D 36	D 36
	String beans...	5.6	Acid	N/1	0	0	0	6.0	Acid	N/1	0	0	0	5.8	Acid	N/1	0	0	0
	Corn.....	6.6	Acid	Abn.	D 12	D 12	D 12	6.8	Acid	Abn.	D 12	D 12	D 12	6.6	Acid	Abn.	D 12	D 12	D 12
	Spinach.....	5.6	Acid	N/1	S 12	S 60	S 48	5.4	Slight acid	N/1	D 24	D 60	S 24	5.4	Slight acid	N/1	S 12	0	S 12
	Peas.....	6.4	Putrid	Abn.	D 12	D 24	D 60	6.6	Putrid	Abn.	D 36	D 24	D 36	6.0	Putrid	Abn.	D 24	D 36	D 36
	String beans...	5.4	N/1	N/1	S 12	S 12	S 12	5.6	N/1	N/1	S 12	S 12	0	5.5	N/1	N/1	S 12	S 12	0
10-12 C.	Peas.....	6.6	N/1	N/1	S 12	S 12	0	6.4	N/1	N/1	S 12	0	0	6.5	N/1	N/1	0	0	0
	String beans...	5.6	Acid	N/1	0	0	0	5.8	Slight acid	N/1	0	0	0	5.8	N/1	N/1	0	0	0
	Corn.....	6.6	Slight acid	N/1	S 12	S 24	0	6.8	Slight acid	N/1	S 12	S 24	0	7.0	Slight acid	N/1	S 12	S 24	0
	Spinach.....	5.5	Slight acid	N/1	S 12	S 12	S 12	5.8	Slight acid	N/1	0	0	0	5.6	Slight acid	N/1	0	0	0
	Peas.....	6.4	N/1	N/1	0	0	0	6.2	N/1	N/1	0	0	0	6.4	N/1	N/1	0	0	0
	String beans...	5.4	N/1	N/1	0	0	0	5.6	N/1	N/1	0	0	0	5.6	N/1	N/1	0	0	0

S indicates animal sick; D, dead.

was evident somewhere earlier in the cultures incubated at 25 C., but once this initial lag period was overcome there was no further noticeable difference.

3. The different vegetables, although inoculated with the same number of organisms, presented marked differences in their gross appearances, as well as in their ability to yield a potent toxin. Uniformly, corn yielded the most potent toxin; in a few instances, in which the minimum lethal dose was determined, it was found that 0.25 c c. of a 1 : 5,000 dilution regularly killed experimental animals within three days. Sufficient toxin to kill white mice in 12 hours was produced in the corn incubated at 20 C. and 25 C. for only 72 hours. Even in the highest dilutions there was no diminution in this toxicity during the entire period of 5 months' observation. Macroscopic changes in the corn, however, were not marked. As incubation proceeded, the grains in the lower portions of the tubes presented a somewhat mushy appearance, with some darkening. After two weeks this had increased to a point at which the normal granular appearance had practically disappeared in the lower part of the tube, with little noticeable change in the upper portions. The corn presented a decidedly butyric odor after 7 days, but no odor of putrefaction; gas production was sufficient to cause bulging of the tops of the cans and lifting of paraffin seals. Lethal doses of toxin in the high dilutions were present before any gross changes could be detected. The appearance of the corn incubated at 20 C. and at 25 C. was similar. No changes in appearance of the corn incubated at 10 C. were noticed even after a period of 5 months.

Peas also were capable of yielding a potent toxin at 20 and 25 C., although not to the same degree as the corn, nor was toxin produced at the same rate. Peas gave evidence of the most marked decomposition of any of the vegetables used. They became decidedly "mushy" in consistency, the entire seed disintegrating. There was much darkening of the medium, and abundant gas production. The odor emitted from this vegetable was not that of a simple acid production, but was decidedly putrid. Bacteriologic examination failed to show any contamination. The picture of seed decomposition was in evidence before sufficient toxin had been produced to be fatal to the experimental animals.

The string beans yielded much less toxin than either corn or peas. While the mice showed evidence of some intoxication when they received injections with beans incubated at both 20 C. and 25 C. for 72 hours, no animals died until they received injections with material incubated for 5 weeks. No toxin sufficient to kill mice was produced in string beans

incubated at 20 C., and the presence of toxin in demonstrable quantities in such material incubated at this temperature was evident only after long periods of incubation. The appearance and odor of the beans remained practically normal. As incubation proceeded, a mild odor of butyric acid could be detected. It is doubtful if this was marked enough to attract attention if one were not specifically looking for it.

The spinach presented little change in appearance, and, with the exception of a slight odor of acid in some of the cans, nothing indicative of contamination with Cl. botulinum could be detected. The quantity of toxin produced was not great. A few of the mice that received injections became ill, but none died even when the largest quantities were given.

4. No marked differences could be noticed in the toxin production or degeneration of the mediums in those vegetables that were kept in the original containers and those in the 50 gm. samples in the pyrex tubes rendered anaerobic by sealing with paraffin. The amount of air entering at the time the cans were opened apparently did not change the oxygen tension sufficiently to exert any influence on the conditions necessary for growth of Cl. botulinum.

5. As will be noted in table 1, the acidity of the spinach and string beans was quite high, the initial P_H being 5.8 and 5.6, respectively. Whether this accounts for the slow growth and the small amount of toxin production was not determined, although it offers a possible explanation.

CONCLUSIONS

Temperatures of 20 C. and 25 C. are equally suitable for the growth of Cl. botulinum and for the production of botulinus toxin in such vegetables as corn, string beans, peas, and spinach. The rate of development is somewhat more rapid at 25 C. than at 20 C., although in each case the toxicity eventually reaches the same level.

A temperature of 10 to 12 C. is not suitable for growth of Cl. botulinum nor for the production of a potent toxin, even when incubation is extended to 5 months.

Death of the organism does not occur at any temperature used, even after 3 months' incubation. Even in those mediums in which no evidence of growth or toxin production could be detected, transplants to a suitable medium, such as beef heart, resulted in abundant growth of the organism with normal toxin production.

Disintegration of the medium parallels the production of toxin, although in some instances lethal doses of toxin may be present with practically no noticeable changes in the gross appearance or in the odor.

The four vegetables used ranked as follows in the rate of toxin production, and in the quantity of toxin produced: corn, peas, string beans, spinach.

The high initial acidity of the beans and spinach may be the responsible factor for the slight growth and toxin production in these mediums.

PULMONARY INFECTIONS BY FRIEDLÄNDER'S BACILLUS

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Infection of the lungs by Friedländer's bacillus has received scant study. A fairly comprehensive review of the English and French literature and of standard works [1] reveals few references to this organism. Sisson and Thompson [2] published an account of 33 cases from the literature, and added four of their own. The present article presents the results of a study of eighteen cases of pulmonary infection by Friedländer's bacillus.

MATERIAL

This material was collected from the necropsy service of the Philadelphia General Hospital, with one case from the Bryn Mawr Hospital. In three cases B. mucosus was found only in the blood cultures made at necropsy, and there is some question as to the propriety of including these cases. However, the accord between the antemortem and postmortem cultures in other cases, and the fact that this bacillus is not a common contaminating organism, seemed to justify their inclusion. In 10 instances the bacillus was found in pure culture, in 1 practically so. It is from these 11 cases that conclusions are chiefly drawn.

CLINICAL NOTES

Of the 18 cases 15 occurred in males and 3 in females with an average age of 52 years. The age incidence and case mortality in Zander's [1] epidemic is given in table 1.

For the entire series (Zander) the mortality was 36%. In my series every case observed has ended fatally. As to incidence, Sisson and Thompson [2] quote Weichselbaum as believing that about 5.5% of all croupous pneumonias to be due to B. mucosus, and they place the incidence between 5 and 10%. These figures seem too high, at least in this country, as there were found in the American literature records of bacteriologic studies of 2,711 cases of pneumonia of all types—exclusive of the tuberculous—in 15 only of which Friedländer's bacillus was

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[1] Aschoff: Pathologische Anatomie, 1923, 2, p. 152. Kaufmann: Pathologische Anatomie, 1922, p. 299. MacCallum: Textbook of Pathology, 1921, p. 255. Adami and Nicholls: Principles of Pathology, 1909, 2, p. 295. Stevens: The Practice of Medicine, 1922, p. 593. Letulle and Bezançon: Ann. de méd., 1922, 12, p. 1. Zander: Deutsch. med. Wchnschr., 1919, 45, p. 1180. Leon and Menville: Bull. et mém. Soc. méd. d. hôp. de Paris, 1924, 48, p. 286. Menetrier, Bertrand and Fontaine: Ibid., p. 114. Cassaude, Joltrain and Surmont: Ibid., p. 148. Lereboullet and Donoyelle: Ibid., p. 226. Brouardel, Renard and Bonnot: Ibid., 1923, 47, p. 990.

[2] Am. Jour. Med. Sc., 1915, 150, p. 713.

found (0.55%).[3] My series does not furnish adequate statistics as bacteriologic studies were not made on all types of pneumonia during the period covered.

The duration of illness varied from 1 day to 5½ months. Subjective and objective symptoms were not different from those ordinarily found in acute and chronic pulmonary infections. However, as compared to clear-cut pneumonia of pneumococcic type the course of the disease was such as to class most cases as atypical. The temperature curves in cases with pure cultures were irregular, and decidedly lower than would be expected, the average maximum for the six acute cases being 102 F. The average temperature for the six was appreciably lower than this, normal and subnormal readings being recorded frequently, especially near death. One case just prior to death had an axillary temperature of 104 F. There was a moderate leukocytosis, averaging 15,000, with 86%

TABLE 1

Age Incidence and Mortality in Zander's Epidemic

Age	Cases	Case Mortaltiy, per Cent.
17-27	246	24.4
28-38	103	32
39-57	41	51.2

of polymorphonuclears in cases with unmixed infection. Leukopenia and normal counts were common, however, the high average being due to one case with several counts over 30,000. There was the usual febrile albuminuria. Abscess formation was not accompanied by foul odor of the breath, except in one case (case 11). Letulle[1] remarked this fact in his case, and contrasted it to another of gangrene of the lung.

In the light of all the available evidence these cases seemed best grouped as follows: terminal lobular pneumonia, 9; chronic suppurative pneumonitis, 3; primary pneumonia, chiefly lobar, 6.

BACTERIOLOGY AND ANATOMY

Of the nine cases of terminal lobular pneumonia, 3 showed Friedländer's bacillus in pure culture. Two of these (1 and 2) at necropsy revealed nothing to distinguish them from ordinary bronchopneumonia. Both were of questionable duration, undoubtedly brief, as the pneumonia was not discovered clinically. Neither had abscess formation. In case 3, the patient, who was a typical instance of cardiac decompensation, developed vague pulmonary signs 6 days before death.

[3] Cole: N. Y. State M. J., 1919, 19, p. 253. Locke, Thomas and O'Hara: Boston Med. & Surg. J., 1920, 183, p. 480. Chickering: Ibid., 1919, 181, p. 679.

At necropsy there was an abscess at the right apex, the pus from which contained no tubercle bacilli, but gram-negative encapsulated bacilli. The heart blood contained a pure culture of B. mucosus. The microscopic picture did not differ from that to be described later. Two patients (cases 4 and 5) had associated lesions of pulmonary tuberculosis, apparently a not uncommon occurrence. The

Fig. 1 (Case 6).—Abscess with fleshy trabeculae from which Friedländer's bacillus was recovered in pure culture at necropsy.

suppuration and abscess formation of the Friedländer's infection was easily distinguished from the older tuberculous lesions. In case 6 pulmonary symptoms were in part due to metastatic carcinoma, and 3 months does not accurately represent the duration of the infection. This was of sufficient standing, however, to produce an abscess without definite walls, 6 by 7 cm., filled with a thin,

greenish, purulent material. Case 7, although a primary pneumonia, is listed here as a probable example of secondary invasion of a pneumococcic pneumonia by B. mucosus. Case 8, although one of mixed infection, showed the bronchial mucosa covered with the characteristic sticky, mucoid exudate. The cases of mixed infection are included in the list as evidence that such do occur, and so to emphasize the care that should be exercised in selecting material in order to exhibit the characteristic changes of this infection.

The three cases of chronic suppurative pneumonitis are of special interest because of their similarity to pulmonary tuberculosis. In each case this lead to such a diagnosis both clinically and at necropsy. Cases 10 and 11, in which Friedländer's bacillus was found in pure culture, deserve brief individual mention.

Case 10.—A white man, aged 56, had been admitted twice previously to the wards for tuberculosis of this hospital. He had complained at various times of hoarseness, cough, expectoration and loss of weight and strength. Examination showed involvement of the lung at the right subclavicular region, and posteriorly from the spine of the scapula to the ninth rib. There was a friction rub over this latter area. There was clubbing of the fingers. On his last admission signs of a cavity were found at the right apex, with surrounding consolidation. Three examinations of sputum showed no tubercle bacilli. The maximum temperature was 100.2 F., just prior to death; the pulse rate was between 90 and 100; the respirations about 25. The entire duration of the illness was about one year.

At necropsy the left upper lobe was the seat of a soft, confluent reddish-gray consolidation, and the lower lobe showed a few smaller although similar areas. The upper and lower lobes of the right lung contained a few small, grayish, ill-defined areas, which were considered miliary tubercles. The lower lobe on this side showed, in addition, larger soft areas, and a moderate dilatation of the bronchioles with thickened walls, a chronic cylindrical bronchitis. The heart blood contained B. mucosus in pure culture. Stained sections revealed an acute pneumonic process with large irregular masses of necrotic material poorly marginated. Innumerable gram-negative bacilli were seen in such areas, in the solid lesions, and in the bronchial walls. There was no suggestion of tuberculosis. Although the evidence here is not conclusive the case suggests a chronic bronchitis due to infection by Friedländer's bacillus, terminating in an acute pneumonic dissemination of this organism.

Case 11.—A white man, aged 50, had a previous history of pleurisy. Five months before admission he was treated for empyema by rib resection. At the time of the present illness, he complained of cough, purulent expectoration, pain in the chest, dyspnea, night sweats, hoarseness and loss of weight and strength. Examination showed considerable involvement of the entire left lung and some lesion at the right apex. This was confirmed by the roentgen ray and a diagnosis of chronic pulmonary tuberculosis with pleurisy was made. A foul odor of the breath was noted. There was clubbing of the fingers. Five examinations of sputum showed no tubercle bacilli. The temperature was 101 at the highest, the pulse rate 90 to 120, respiration 21 to 32. The patient grew weaker and died, after an illness of 5½ months (15 days in the hospital).

At necropsy both pleurae were obliterated by dense fibrous adhesions. The right lung weighed 810 gm. The upper lobe exuded much greenish, fibrinous material; the middle lobe was emphysematous; the lower lobe contained areas described as a "fair number of miliary and conglomerate tubercles." The left lung weighed 725 gm. It was densely fibrotic and practically airless, and there were many small cavities in both lobes. The necropsy record states that a "few definite

tubercles were noted." The heart blood contained B. mucosus in pure culture. Stained sections showed absolutely no evidence of tuberculosis. Instead there was a dense hyaline fibrous tissue enclosing purulent and necrotic collections of various sizes. Elsewhere pneumonic alveoli contained fibrin, pus and desquamated cells of the alveolar walls. Some of the abscesses possibly were bronchial, as traces of walls were suggested here and there. The necrotic areas contained many gram-negative bacilli; the pneumonic areas fewer.

This case, like the previous one, would be more convincing had there been antemortem demonstration of Friedländer's bacillus. It is

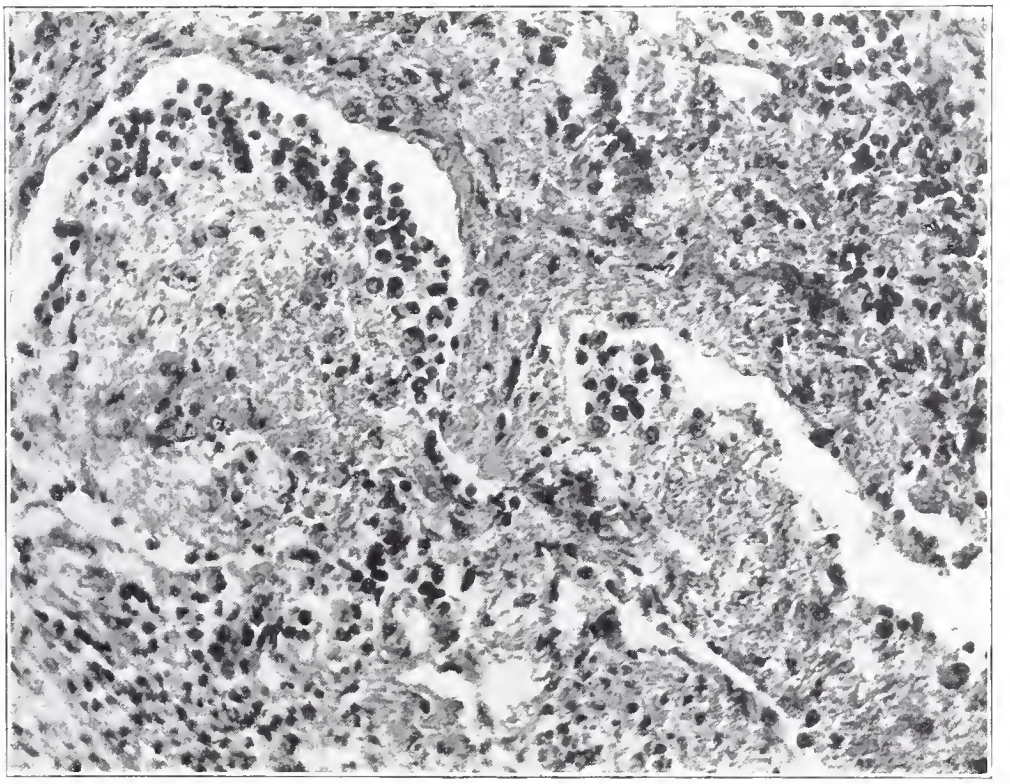

Fig. 2 (Case 11).—Chronic suppurative pneumonitis showing fibrous tissue and abscesses.

believed, however, that these two cases are justly presented as possibly representing a true chronic suppurative pneumonitis due to B. mucosus. We have found no record of a similar observation.

The cases of primary acute pneumonia, which constitute the most important part of this report, were as follows:

CASE 13.—A white man, aged 66, after exposure to rain about one week previous to admission, developed a severe pain in the left chest, made worse by a hacking cough. He was weak and dyspneic. On admission there was imperfect expansion of the lower part of the left side of the chest, and a pleural friction

and fine râles to the left of the cardiac area. Bronchial breathing was present over several areas in this lung. The type of sputum was not recorded. Typhoid facies was present, an da provisional diagnosis of typhoid fever, influenza, or tuberculosis was made. Three days later more definite signs of consolidation appeared in the upper lobe, and a culture of the sputum showed a practically unmixed growth of Friedländer's bacillus. The patient developed severe diarrhea, and died 18 days after onset. The temperature was irregular, varying from 102.4 F. to normal in 24 hours. The pulse averaged 118, respiration 30. The leukocytes numbered 14,900 with 86% polymorphonuclears. Eight cultures of sputum all showed B. mucosus, and rarely a few other organisms.

The left lung weighed 1440 gm.; the pleura was covered with a purulent exudate. The upper lobe was almost completely solid. The cut surface was reddish gray, granular and moist, and on pressure exuded a large amount of gelatinous, sticky material. At the apex was a cavity, 7 by 10 cm., without definite walls, containing a blood stained purulent material. The right lung was normal.

Friedländer's bacillus was recovered in pure culture from the left lung and from the heart blood. Stained sections showed an acute pneumonic process with early abscess formation. The exudate was fibrinous, with many large round cells and a few leukocytes, and contained many gram-negative bacilli. There were no other essential lesions.

Case 14.—A white man, aged 63, was admitted to the Bryn Mawr Hospital with a strangulated inguinal hernia, which was immediately operated on under local anesthesia. He progressed nicely for 3 days, then developed a cough, dyspnea, cyanosis and râles in both lungs. Consolidation of the upper right lobe and collapse of the lower lobe on this side were diagnosed. Death occurred 10 days later. The temperature varied from 96 to 100 F.; the pulse rate averaged 100, respiration 34. The leukocytes numbered 12,600 per cubic millimeter.

At necropsy the pleura on the right was adherent in places, forming pockets containing a yellow, sticky, mucoid exudate. There were only 2 lobes in this lung. The upper was voluminous and solid, and on section presented a pinkish-gray, moist, granular surface studded with numerous abscesses from 1 to 20 mm. in diameter. These and the cut surface exuded a material similar to that in the pleura. An abscess, 3 cm. in diameter, occluded the bronchus to the lower lobe, which was partially collapsed. The left lung showed an early bronchopneumonia and an old tuberculous lesion at the apex. The pericardium contained considerable exudate, similar to that seen elsewhere. Cultures from the pericardium and the right lung resulted in a pure growth of Friedländer's bacillus. Stained sections showed a fairly uniform consolidation composed of leukocytes and fibrin. Both early and well formed abscesses were noted. These had no well defined walls, but merged into the pneumonic areas.

Case 15.—A white man, aged 38, was admitted to the hospital while under the influence of alcohol. A history of onset 2 days previously with chills, followed by cough and pain in the right side of the chest, was elicited. Examination showed a well developed man, apparently acutely ill, dyspneic and cyanosed. There were classical signs of consolidation at the right pulmonary apex. The type of sputum was not recorded. Two days later the middle lobe was involved. Signs indicating resolution at the right apex were noted just prior to death. The duration of the illness was 6 days. The temperature was irregular, from 103 to 97 F.; at death it registered 104 by axilla. The pulse averaged 120, respiration 40. Leukocytes on the first 3 days after admission were 5,600; 11,100 and 8,500 per cubic millimeter, the polymorphonuclears averaged 86%. Sputum injected into a mouse gave no pneumococci, but a pure growth of B. mucosus.

TABLE 2

SUMMARY OF CASES

No.	Clinical Diagnosis	Bacteriology: During Life	Bacteriology: At Necropsy	Duration in Days	Abscess in Lung
		Terminal Lobar Pneumonia			
1	Myocarditis, diabetes	.....	B. mucosus in heart blood	?	0
2	Mitral regurgitation chronic nephritis	B. mucosus in sputum	B. mucosus in heart blood	?	0
3	Myocarditis, nephritis, syphilis, pulmonary congestion, hydrothorax	.....	B. mucosus in heart blood, encapsulated gram-negative bacilli in smear of lung	6	+
4	Pulmonary tuberculosis, syphilis	.....	B. mucosus in heart blood and kidney; B. coli, gram-negative bacilli (encapsulated) in lung and kidney	?	+
5	Abscess of lung	B. mucosus, M. catarrhalis in sputum	B. mucosus in lung	120	+
6	Carcinoma of colon, metastatic carcinoma of the lung, pneumonia	.....	B. mucosus in lung, streptococcus in heart blood	?	+
7	Lobar pneumonia, meningitis	B. mucosus, pneumococcus, type 4, in sputum	B. mucosus, pneumococcus type 4, streptococcus in lung; streptococcus in heart blood	25	0
8	Chronic myocarditis, diabetes, senility	.....	B. mucosus, streptococcus in heart blood	?	0
9	Cerebral thrombosis, bronchopneumonia	.....	B. mucosus in heart blood; B. mucosus, B. influenzae, streptococcus in lung	1 or 2	0
		Chronic Suppurative Pneumonitis			
10	Pulmonary tuberculosis, syphilis, cystitis	Negative for B. tuberculosis (3 examinations)	B. mucosus in heart blood	365	+
11	Chronic pulmonary tuberculosis, pleurisy	Negative for B. tuberculosis (5 examinations)	B. mucosus in heart blood	165	+
12	Myocarditis, pulmonary tuberculosis	Negative for B. tuberculosis (3 examinations)	B. mucosus, pneumococcus type 2, B. influenzae from lung; pneumococcus type 2 in heart blood	42	+
		Primary Lobar Pneumonia			
13	Pneumonia, cirrhosis of liver	B. mucosus in sputum	B. mucosus in heart blood and in lung	18	+
14	Strangulated inguinal hernia, pneumonia	.....	B. mucosus in lung and pericardium	10	+
15	Lobar pneumonia	B. mucosus in sputum and in blood culture	B. mucosus in heart blood	6	+
16	Lobar pneumonia, pleurisy	B. mucosus in lung puncture and blood culture; B. mucosus, streptococcus, staphylococcus in sputum	.....	4	+
17	Myocarditis, pneumonia	.....	B. mucosus in heart blood and empyema	15	+
18	Lobar pneumonia, alcoholism	.....	B. mucosus in heart blood and lung	4	0

At necropsy there were a few cc. of turbid fluid in the right pleural cavity. There was also a marked chronic fibrous pleurisy. The right upper lobe and part of the middle lobe were entirely consolidated. The cut surface appeared to be a uniform dark gray, and on being scraped exuded a thick purulent material which adhered to the knife. The bronchioles were filled with a similar material. The lower lobe was edematous and congested. Stained sections showed recently formed small abscesses. The left lung showed emphysema of the upper lobe and congestion of the lower. There were no other essential lesions. Friedländer's bacillus was recovered from the heart blood in pure culture. Stained sections were generally similar to those of the previous case, but the majority of the cells in the exudate were "round." Gram-negative baccilli were demonstrated.

Case 16.—A white woman, aged 31, gave a history of sudden onset of illness 24 hours previous to admission, with chills, cough and pain in the right side. She was dyspneic and apparently acutely ill. Crackling râles were present over both lungs, and a dull percussion note and bronchial breathing on the right side. The heart sounds were of poor quality. The temperature was 101.3 F. on admission, falling steadily to 97 at death. The pulse averaged 120, the respiration 40. There were 6,500 leukocytes with 87% polymorphonuclears. Cultures taken from the blood and from the lung by puncture showed a pure growth of Friedländer's bacillus. From the sputum this organism was recovered with S. viridans, S. hemolyticus and staphylococci. The day before death signs at the right apex suggested a cavity. The duration of the illness was 4 days.

The right lung weighed 1,350 gm. The pleura was thickened. The upper lobe was completely consolidated, and on section was seen to be mottled gray, brown and red. An abundant gelatinous material exuded on pressure. Stained sections showed recently formed small abscesses. The left lung weighed 575 gm. In the lower lobe were small, gray consolidated areas from which a similar exudate escaped. There were no other essential lesions. Stained sections were similar to those previously described. Gram-negative bacilli were demonstrated.

Case 17.—A colored woman, aged 68, developed a cough, a slight, frothy sputum, pain in the right side of the chest, weakness and sweating 8 days before admission. When first seen these symptoms were present, and in addition dyspnea and an abundant brownish-red sticky sputum. There was pain in the right lower axillary region, which was made worse by coughing. Expansion of the right chest was limited. Posteriorly, over the upper and middle lobes, there were signs of consolidation, and in the axilla a friction rub. Râles were heard at both bases. The patient grew steadily worse, and died 15 days after the onset. The temperature varied from 101 to 97 F., the pulse rate from 140 to 80, and respirations from 25 to 55, the lower records all occurring near death.

At necropsy the right pleura was adherent, and contained about 300 cc. of pus. The right lung weighed 1,050 gm., and was entirely consolidated. The cut surface was reddish gray, and exuded pus. A large cavity occupied the lower part of the upper lobe and the upper part of the lower lobe. It contained brownish pus and was crossed by a number of fleshy trabeculae. The left lung grossly was normal, except for a few pleural adhesions. There were no other essential lesions. Cultures from the heart blood and from the pus in the pleural cavity resulted in unmixed growth of Friedländer's bacillus. Stained sections showed some fibrosis associated with old anthracotic areas. An acute pneumonic process with abscess formation and purulent exudate constituted the chief feature. Gram-negative bacilli were shown, most numerous in the pus, but in all other parts also.

CASE 18.—A white man, aged 64, gave a history of heavy drinking and of having hurt the left side of his chest in a fall. Three days before admission he became severely ill, with pain in the left side of the chest near the heart, and increasing dyspnea, cough and a mucoid expectoration, not bloody. On examination the patient was extremely dyspneic, cyanotic and apparently moribund.

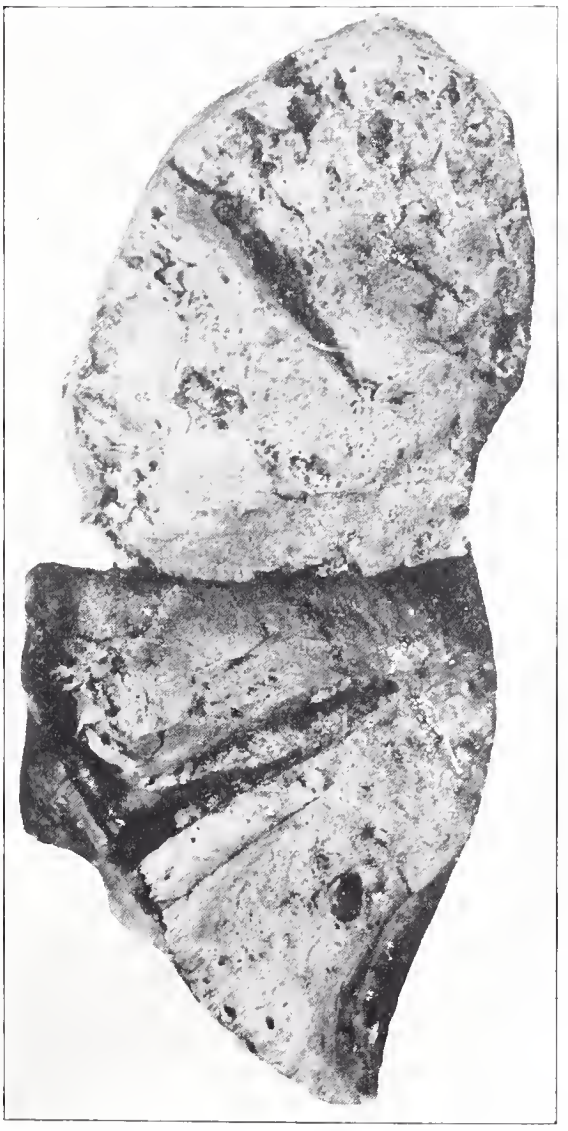

Fig. 3 (Case 14).—Lobar pneumonia of Friedländer's bacillus origin, showing consolidation and multiple abscesses (section of upper lobe).

Evidence of consolidation was found at the left pulmonary base, and a friction rub was heard at this point. The heart sounds were of poor quality. Death occurred 4 days from the onset of the illness. The temperature varied from 102.2 to 101.8 F.; the pulse rate averaged 140, respiration 45.

The left lung weighed 1,080 gm. The pleural surface was dull and finely granular, and over the lower lobes was grayish red and black. The upper lobe was edematous, the lower contained yellowish areas of consolidation from which a purulent exudate was expressed, surrounded by grayish-red zones. Elsewhere there were hemorrhage and edema. An old tuberculous lesion appeared at the apex. The bronchial mucosa was covered with a purulent exudate. The right lung was congested. Friedländer's bacillus was recovered in pure culture from the left lung and heart blood. Microscopic sections showed early red patchy pneumonia with little pus, but no abscesses. Giemsa's stain showed a few bacilli in the bronchioles. These were not demonstrated by Gram's method. There were no other essential lesions.

This series seems to demonstrate a progressive lesion. In the earliest stage, of 2 or 3 days' duration, the lobular consolidation reveals nothing characteristic. Soon after this the exudate acquires the characteristic sticky, mucoid appearance, and abscesses develop. The picture is then sufficiently striking to constitute a pathologic entity, easily recognizable on postmortem examination. We have not seen any other bacterium produce a similar exudate, and we believe this feature to be pathognomonic. Destruction of lung tissue seems almost as characteristic. Abscesses occurred in 12 of the 18 cases, the earliest being of 4 days' duration. Of the 6 cases without abscess, one was of 4 days' duration, the others having existed a shorter, or questionable, time. Apparently, then, all such infections result in destruction of tissue in 4 days, or shortly thereafter. Case 7 is a possible exception. The amount of destruction bears a definite relation to the age of the lesion and seems to progress as long as the patient survives. The absence of definite abscess wall is striking. The chronic type of B. mucosus infection of the lung, as described, is apparently chronic from the beginning, terminating by an acute pneumonic dissemination of the organism. The evidence for this type of lesion is not considered conclusive. However, the instances given are suggestive enough to raise the hope that others of similar nature will be found. It would seem worth while to examine certain tuberculous patients, in whom tubercle bacilli cannot be found, for Friedländer's bacillus.

It was hoped that the microscopic sections would reveal a picture as characteristic as the gross. This, however, was not the case. Neither hematoxylin and eosin, Gram or Giemsa stains or the Mallory connective tissue stains permitted recognition of that element of the exudate imparting the gelatinous appearance. Consolidated areas varied in the amount of serum, fibrin, pus cells, round cells and necrotic matter, thus differing from a pneumococcic lobar pneumonia. The "round" cells were of interest. In cases 1 and 13 they were as numerous as the

polymorphonuclears, and in case 15 constituted about 99% of all cells seen. Sisson and Thompson[2] considered these to be cells from the alveolar walls. Phagocytosis of Friedländer's bacilli, though not studied in all cases, was sufficiently striking in sections stained by the Giemsa method. The monocytes seemed more phagocytic for these organisms than the polymorphonuclears, an observation made in a study of another lesion associated with this bacterium.[4] This same study makes one hesitate to say that these cells are derived from the alveolar walls, though I am not prepared to classify them otherwise. At any rate, they are not especially characteristic of Friedländer's bacillus infection of

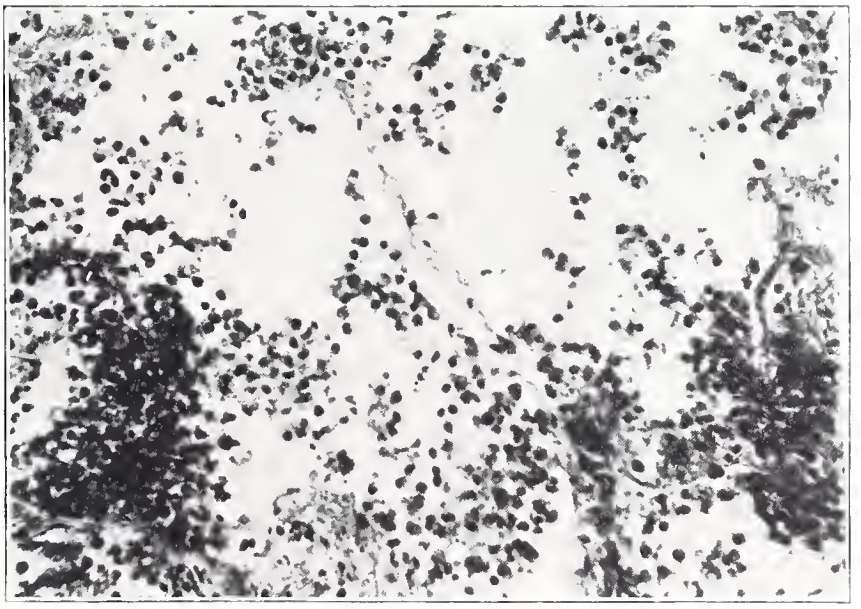

Fig. 4 (Case 16).—Primary lobar pneumonia, showing early abscess formation with solution of alveolar walls.

the lung, in the majority of cases of which polymorphonuclear cells predominate. The most striking feature of the microscopic lesions was the marked destruction of tissue. In early lesions the pulmonary alveolar walls were seen to swell, stain faintly, and then dissolve without the usual demarcation between necrotic and less diseased tissue. In places tissue death en masse could be seen similating somewhat the change in infarction. Even the pus cells in the abscesses were, for the most part, undergoing dissolution. Only in the chronic cases was there any reactive border to the abscesses. Undoubtedly, B. mucosus possesses an extremely powerful cytolytic toxin.

[4] Randall, Small and Belk: Surg., Gynec. & Obst., 1922, 34, p. 717.

The acute primary pneumonias caused by this organism apparently originate as lobular lesions, which quickly coalesce. In all important respects, both clinical and pathologic, they are essentially lobar, and should be so considered. This is undoubtedly a disease sui generis, and in support of this statement the present report is submitted.

SUMMARY

Eighteen cases of infection of the lung by B. mucosus were studied, and a provisional report is made of a chronic pulmonary lesion caused by this organism. Six cases of lobar pneumonia due to B. mucosus are reported in detail, with references to 38 cases in the literature.

THE GERMICIDAL PROPERTIES OF SOAP

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The work of two previous publications [1] has been extended to include observations on the effect of various soaps on the meningococcus, the gonococcus, and several organisms of the intestinal group.

The technic is based on the Hygienic Laboratory method for the determination of phenol coefficients, and is described in detail in the former papers. Stock strains of the meningococcus (type 1) and of the gonococcus, obtained from the Army Medical School, were found to grow in "hormone" broth with sufficient luxuriance for carrying out the tests. The intestinal bacteria were grown in beef extract broth, P_H 7.4.

EXPERIMENTAL RESULTS

The results with chemically pure soap are shown in table 1. There was some irregularity in the tests made on different days, and occasionally there was "skipping" of a tube, that is, a tube failed to show growth, although the next stronger dilution showed growth. The figures recorded are for strengths that failed to show growth on any

TABLE 1

Killing Strengths of Soaps for the Meningococcus and Gonococcus

20 C.

	Meningococcus		Gonococcus	
	2½ Min.	15 Min.	2½ Min.	15 Min.
Na laurate	N/320	N/320	N/640	N/640
Na myristate	N/640	N/1280	N/1280	N/1280
K palmitate	N/80	N/160	N/640	N/1280
K stearate	N/160	N/160	N/640	N/640
Na oleate	N/320	N/640	N/2560	N/2560
Na linolate	N/640	N/640	N/5120	N/5120
Phenol	1%	1%	½%	½%

occasion. The tests were repeated at least twice, and usually three times. It can be seen from the table that there is no marked selective activity of any of the soaps for the meningococcus and gonococcus, as was shown in the case of the pneumococcus and streptococcus. Such

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[1] Walker, J. E.: Jour. Infect. Dis., 1924, 35, p. 557; 1925, 37, p. 181.

differences as exist between the soaps are possibly related to the technical difficulties just mentioned, and I am not inclined to stress them. The gonococcus is, however, evidently more susceptible to the action of soap than the meningococcus.

The meningococcus, as the more resistant of the two organisms, was further tested against 4 commercial soaps, with the results shown in table 2.

TABLE 2

KILLING STRENGTH OF COMMERCIAL SOAPS FOR THE MENINGOCOCCUS

20 C.

	2½ Min.	15 Min.
White floating	1:640	1:2,560
Scented toilet	1:640	1:5,120
Brown bar	1:640	1:2,560
Sapo mollis (U.S.P.)	1:640	1:1,280

In addition, sodium resinate prepared in the laboratory was found to kill the meningococcus in the dilution of 1 : 400 in 2½ minutes, and the gonococcus in 1 : 1,600 in the same time. The 15 minute killing strength was the same.

The Shiga and Flexner dysentery bacilli and paratyphoid A and B were tested against chemically pure soaps. The results (table 3) show that the resistance of these organisms is almost identical with that formerly [2] reported for the typhoid bacillus.

TABLE 3

15 MINUTE KILLING STRENGTH

20 C.

	B. dysenteriae Flexner	B. dysenteriae Shiga	Paratyphoid A	Paratyphoid B
Na laurate	N/20	N/20	N/40	N/10
K myristate	N/20	N/40	N/40	N/20
K palmitate	N/80	N/40	N/40	N/20
K stearate	*	N/20	*	*
Na oleate	—†	—	—	—
Na linolate	—	—	—	—
Phenol	1%	1%	1%	1%

* N/20 did not kill; N/10 formed too firm a gel at 20 C. to be tested.
† The minus sign indicates that the strongest solution tested (N/5) did not kill.

Previous work [3] has shown that soap prepared from cocconut oil is more germicidal against the typhoid bacillus than soap prepared from any other base. Soap prepared from coconut oil is available com-

[2] Walker, J. E.: Jour. Infect. Dis., 1924, 35, p. 557.
[3] Ibid., 1925, 37, p. 181.

mercially in the form of salt water soap, since this is prepared from coconut oil or palm kernel oil.[4] Such a soap, stated by the manufacturers (Colgate & Company) to be made exclusively of coconut oil, was tested against the typhoid bacilli. The 15 minute killing strength at 20 C. was 1:80, exactly the same as obtained for coconut oil soap prepared in the laboratory.

Further, in the type of hand washing experiment described in the previous paper,[3] using B. coli as the test organism, this soap was found invariably to sterilize the hands (for B. coli) in 1 minute whenever a fairly stiff lather was made. This was accomplished by none of the other commercial soaps tested, and of the soaps prepared in the laboratory, only by coconut oil soap.

Pure coconut oil soap is, however, probably too irritating to the skins of most people to be used constantly; 25% of coconut oil is about the greatest amount that can be added to toilet soap base,[4] and such a soap ("white floating soap") has already been shown to possess only limited germicidal value for intestinal bacteria as tested with B. coli.

CONCLUSIONS

The meningococcus is killed in 2½ minutes by N/80 to N/640 solutions (0.4% to 0.04%) of the soaps of the fatty acids ordinarily present in soap bases; 1% phenol is required to kill under the same condition.

The gonococcus is killed in 2½ minutes by N/640 to N/5,120 solutions (0.04% to 0.006%) of the same soaps; 0.5% phenol killed under the same condition.

Sodium resinate also possesses marked germicidal activity toward these two organisms.

The meningococcus, on being tested with four commercial soaps, showed approximately the same degree of susceptibility as would have been anticipated from the action of the chemically pure soaps.

The susceptibility of meningococci and gonococci is such that they (along with streptococci, pneumococci, and diphtheria bacilli [1]) will be readily killed by any ordinary soap used with a reasonable degree of care.

The dysentery bacilli and paratyphoid bacilli react to the different soaps in the same manner as previously shown for typhoid and colon bacilli, that is, they are killed by moderate concentrations of the soaps

[4] Simmons, W. H.: Soap, 1919, p. 9.

of the saturated acids, but are completely resistant to the soaps of the unsaturated acids at ordinary temperatures.

The most readily available commercial soap to be used against the typhoid bacilli, paratyphoid bacilli, and dysentery bacilli is salt water soap prepared exclusively from coconut oil.

BIOLOGIC SIGNIFICANCE OF THE SOLUBLE SPECIFIC SUBSTANCES OF PNEUMOCOCCI *

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In the course of our study on the antibody of antipneumococcus serum, it was repeatedly observed in protection tests that mice that had received injections with large doses of serum or with concentrated antibody solutions died while those receiving injections with smaller doses lived. That there is an apparent discrepancy in the protective values of the higher and lower doses of serum or specific precipitates, was early noted by Chickering [1] in his study on the protective bodies in antipneumococcus serum. This investigator observed that increasing the size of the dose of the protective agent may not increase the protection when titrated against a given dose of pneumococci. It has been a question in our minds whether this phenomenon referred to is an inherent characteristic of the antibody, or whether the serum or antibody solution contains two substances, one protective and the other inhibitory to the animal's defense. It has been reported previously [2] that the latter assumption is the likely one. For it was shown that certain samples of type 2 serums when diluted with equal volumes of water yield a precipitate which not only paralyzes the defense of the animal, but in greater amounts produces fatal results. This precipitate has been dissociated and yields both protective and toxic fractions. In the present investigation, the water-soluble specific substance isolated from pneumococci by the method of Heidelberger and Avery [3] is considered one of the possible bacterial products that cause this action of immune serum antagonistic to defense. Indeed, the work of Cole,[4] Bail,[5] Wassermann and Citron,[6] and others, concerning the action of certain soluble substances and the so-called "agressins" in the body

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* This is one of a series of studies carried out in part under a grant by the Influenza Commission of the Metropolitan Life Insurance Company.

[1] Jour. Exper. Med., 1915, 22, p. 248.

[2] Jour. Infect. Dis., 1925, 37, 199.

[3] Jour. Exper. Med., 1924, 40, p. 301.

[4] Ibid., 1917, 26, p. 453.

[5] Arch. Hyg., 1905, 52, p. 272.

[6] Deutsch. med. Wchnschr., 1905, 31, p. 1101.

fluids of infected animals, would indicate that such an hypothesis is not unreasonable.

This study is almost entirely limited to the soluble specific substance of type 2 pneumococci for the reason that we had a larger supply of this type. Lots from types 1 and 3 have been prepared, and preliminary experiments indicate that similar results to those reported here with the type 2 substances are to be expected. It may be stated that, as far as chemical analyses have been made, the lots prepared from types 2 and 3 pneumococci compare favorably with those reported by Heidelberger and Avery. Samples, when tested with a large number of immune serums and antibody solution, showed very high precipitating power, as indicated by titer and specificity, and contained only traces of nitrogen (0.1 to 1% by weight).

INFLUENCE OF SOLUBLE SPECIFIC SUBSTANCE IN VIVO WHEN INJECTED WITH AN ATTENUATED CULTURE

Exper. 1.—The observation that the substance precipitated from immune serum by the soluble specific substance when tested for protective value, displayed in large doses the antagonistic action referred to above,[2] led us to determine the effect of the soluble specific substance itself. The experiments to test toxicity showed that as much as 20 mg. per c c. produced the effect of only a temporary weakening of the mice injected. Accordingly, it appeared possible that the soluble substance in the doses injected acted, not as a lethal agent, but as an agent causing specific paralysis in the animal's defense. To test this assumption, the soluble specific substance was injected together with varying numbers of pneumococci which had become attenuated by growth on plain broth. In the first experiment, shown in table 1, a virulent type 2 culture of pneumococci was attenuated by daily transfer for one week on plain broth. The influence of different amounts of the soluble specific substance was determined by doses ranging from 5 mg. to 0.005 mg. injected into duplicate mice with dilutions of culture of 1 : 10,000 to 1 : 10,000,000. The results of this experiment (table 1) show that this lot of soluble specific substance in some manner aids in producing a fatal infection with a relatively avirulent culture of pneumococci.

A repetition of this experiment is shown in table 2, the only difference being the use of another culture of type 2 organisms attenuated by the same method. The results here again show that the specific substance used in these experiments weakens the defense of the animals.

It will be noted that the degree of activity is not exactly proportional to the amount of specific substance injected; for, as seen in both tables 1 and 2, similar effects were produced in doses of 5 mg. to 0.005 mg. A

TABLE 1

INFLUENCE OF SOLUBLE SPECIFIC SUBSTANCE IN VIVO WHEN INJECTED WITH AN ATTENUATED CULTURE

Dose of Soluble Specific Substance, Mg.	Dilution of Culture			
	1:10,000	1:100,000	1:1,000,000	1:10,000,000
5.0	36 36	36 36	36 60	36 S
0.5	36 36	36 36	36 36	36 S
0.05	36 36	24 36	S S	36 S
0.005	36 36	24 36	36 S	36 S
0.0005	36 36	44 84	S S	S S
Control	36 60	36 36	S S	S S

Numbers refer to hours of survival.

TABLE 2

INFLUENCE OF SOLUBLE SPECIFIC SUBSTANCE IN VIVO WHEN INJECTED WITH AN ATTENUATED CULTURE

Dose of Soluble Specific Substance, Mg.	Dilution of Culture				
	1:10,000	1:100,000	1:1,000,000	1:10,000,000	1:100,000,000
5.0	36 36	24 36	36 36	36 36	44 S
0.5	36 36	36 36	36 36	36 36	S S
0.05	24 36	36 36	36 36	36 60	36 36
0.005	24 24	36 36	36 108	48 108	S S
0.0005	36 36	36 S	36 36	42 S	96 S
Control	36 36	36 S	S S	S S	S S

Numbers refer to hours of survival.

repetition with another lot of type 2 specific substance prepared in the same manner was found to produce the same effects.

The assumption that the results of these experiments represent one phase of the biologic significance of the soluble specific substance

is made with the reservation of the possibility that some toxic material is present in these preparations other than the specific precipitable substance. In the analyses of the preparations used, the final products correspond to those described by Heidelberger and Avery,[3] with the exception of a slightly higher nitrogen content. Moreover, similar results have been obtained by the injection of dissolved pneumococci with attenuated cultures in a manner shown in these experiments. Accordingly, the supposition might be made that the action with the soluble specific substance was due to a toxic protein combined as an essential part of the molecule, or as a contamination, perhaps similar to the nitrogenous fraction of "residue antigens" reported by Mueller, Wayman and Zinsser.[7] However, it should be pointed out that in a dose of 0.005 mg. of the material, which is essentially a complex carbohydrate of a large molecule, the amount of such protein necessarily would be small. Furthermore, the titers of the specific precipitin reactions with the lots prepared were very high (1 : 2,000,000 to 1 : 10,000,000 in serum and 1 : 20,000,000 in antibody solutions). The ultimate answer, however to the question as to what causes the effects given in this experiment, awaits the preparation of the pure specific substance. Whatever the outcome, the fact must be borne in mind that an extremely small amount of specific substance or of some bacterial product may neutralize or destroy the varied manifestations of antibodies.

THE INFLUENCE OF CONCENTRATION OF THE SOLUBLE SPECIFIC SUBSTANCE ON THE PRECIPITATION OF THE PROTECTIVE PROTEIN FROM TYPE 2 SERUM

Exper. 2.—In preliminary experiments it was found that the concentration of the soluble specific substance which gave the best precipitation was between 1 : 2,000 and 1 : 15,000 for the average immune serum. An extension of this observation to include the amount of protective substance precipitated was the object of this experiment. Type 2 serum of high potency accordingly was precipitated with different concentrations of the specific substance (1 : 200, 1 : 2,000 and 1 : 20,000). The precipitate from each sample, the supernatants, the original serum and a precipitate prepared by 1 :5 dilution of the same volume of serum with distilled water, were tested for protective value. The experiment was carried out as follows: An equal volume of an 0.85% sodium chloride solution of the soluble specific substance in the desired concentration was added to each tube of serum, which was thoroughly shaken

[7] Proc. Soc. Exper. Biol. & Med., 1923-4, 21, p. 241.

and incubated for 2 hours in the water bath at 37.5C. The tubes were then put in the icebox and centrifugalized the following morning. The precipitates thus obtained were practically insoluble in neutral salts and were injected into mice as a finely divided suspension in 3% sodium chloride. A summary of the results shown in table 3 is as follows: (1) A concentration of 1:2,000 of the soluble specific substance apparently produced the greatest amount of precipitate, and yet the latter displayed no protective action. With a concentration of 1:200

TABLE 3

PRECIPITATION OF PROTECTIVE SUBSTANCE IN TYPE 2 SERUM BY SOLUBLE SPECIFIC SUBSTANCE

(All mice received injections of 0.5 c c. of culture diluted 1:100)

Dose of Soluble Specific Substance, Mg.	Dilutions of Samples				
	1:5	1:10	1:20	1:40	1:80
Precipitates with 2.5 mg.	S S	S S	36 S	96 S	36 60
Precipitates with 0.25 mg.	68 96	36 60	36 60	66 84	36 36
Precipitates with 0.025 mg.	24 36	S S	S S	36 S	84 96
Supernatants with 2.5 mg.	S S	S S	84 S	36 40	36 36
Supernatants with 0.25 mg.	36 84	21 36	21 24	24 24	20 21
Supernatants with 0.025 mg.	S S	S S	S S	40 S	36 60
Serum control	S S	S S	S S	60 84	60 60
Precipitate by dilution with water	84 84	S S	S S	S S	144 36
		1:10,000,000		1:1,000,000	
Organism control		36 36		24 36	

Numbers refer to hours of survival.

of the specific substance there was less precipitate due to a prozone effect, and about one-half as much protection as with the original serum, while with the lowest concentration (1:20,000) protection was present in the same titer as in the control serum. (2) The supernatant from the precipitate with 1:2,000 soluble specific substance indicates that all of the protective material had been precipitated from the serum at this concentration; with the 1:200 there remained in the supernatant approximately the difference between the amount of protective sub-

stance precipitated and that in the original serum; while in the case of 1 : 20,000 concentration the protective titers of both the supernatant and the precipitate were practically the same. It must be noted, however, that if titrations with less difference in dilutions had been made, closer approximations to the original value might have been found.

The results obtained with the precipitate prepared by dilution of serum with water show that the protective titer in this case, however, was twice that of the original serum. The explanation of this apparent irregularity seems difficult, but its occurrence in this and other studies warrants the assumption that it is due to a toxic or antagonistic substance present in the serum, which is removed in part in the process of precipitation. The foregoing results as to the influence of concentration of the soluble specific substance on the precipitation of the protective bodies from type 2 serum appear almost to be in complete accord with the work of Morgan,[8] who demonstrated that for optimum precipitation from any amount of type 2 antipneumococcus serum there exists an optimum concentration of the soluble specific substance. It is interesting to note that in this investigation also a final dilution of 1 : 2,000 of the reacting substance gave the heaviest precipitate with the immune serum.

EFFECT OF CONCENTRATION OF THE SOLUBLE SPECIFIC SUBSTANCE ON ANTIBODY SOLUTIONS IN VITRO

Exper. 3.—In this experiment a study was made on the effect of the concentation of the specific substance on type 2 antibody prepared by precipitation of serum by dilution with water as was done with serum in the preceding experiment. To a series of equal volumes of a potent type 2 antibody soluble specific substance was added to the desired concentration, and after the usual period of incubation, the samples were diluted 1:5 with cold distilled water. After centrifugalization, the precipitates were made up in 1% sodium chloride to the original volumes of the antibody solutions.

As may be seen from table 4, the antibody control was highly protective, a dilution of 1 : 400 protecting against 0.01 c c. of a type 2 culture. On the other hand, neutralization of protective value occurred by the specific substance, depending on the amount present. It should be pointed out that in the same manner as in the case of serum there was no measurable antagonistic substance in the antibody solution before the specific substance was added; whereas, in the case of the precipitate

[8] Jour. Immunol., 1923, 8, p. 449.

with water plus 1 : 20,000 concentration of the soluble specific substance, a dilution of 1 : 2 failed to protect, while dilutions as high as 1 : 100 and 1 : 200 exhibited definite protection. It will be noted also that all the mice survived that received injections with the soluble specific substance alone in amounts much greater than were used in the precipitation of these samples.

TABLE 4

IN VITRO NEUTRALIZATION OF ANTIBODY SOLUTION BY SOLUBLE SPECIFIC SUBSTANCE

(All mice received injections of 0.5 c c. of culture diluted 1:100)

Dose of Soluble Specific Substance, Mg.	Dilution of Antibody Solution			
	1:2	1:100	1:200	1:400
2.5	S S	84 S	36 36	18 20
0.25	18 18	36 60	18 36	18 18
0.025	18 36	S S	S S	66 72
Control	S S	S S	S S	S S
Control of Soluble Specific Substance	1:2	1:10	1:20	1:40
	S S	S S	S S	S S
	1:10 000,000		1:1,000,000	
Organism Control	24 36		18 24	

Numbers refer to hours of survival.

EFFECT OF HEAT ON THE SPECIFIC PRECIPITATE AND ON THE WATER-INSOLUBLE PRECIPITATES OF TYPE 2 SERUM

Exper. 4.—This experiment was carried out to show the influence of heat on the dissociation of the precipitate obtained by the specific soluble substance and on the antibody obtained by dilution of serum with water. The same general procedure as already described was carried out in preparing these samples. The precipitates prepared by both methods were washed several times with cold distilled water. That obtained by dilution with water was dissolved and made up to the original volume of the serum in 1% sodium chloride and divided into two portions. The precipitate prepared with soluble specific substance was suspended in the same volume of 1% sodium chloride, and after thorough shaking was also divided into two equal parts. One sample of each preparation was then heated at 54 C. for one-half hour and tested as indicated in table 5. In the first place, it can be noted that

the sample of precipitate obtained by dilution of serum with water on redissolving gave the same protective titer as the original serum, and after heating showed a loss in titer similar to results reported elsewhere.[9] The results with the precipitate prepared with the soluble specific substance show the possibility of dissociation and liberation of the protective protein by heat. However, the results given here show a much greater degree of dissociation by this means than was found in many of our other experiments, a report of which will be made in another publication.

TABLE 5

Effect of Heat on the Specific Precipitate and on the Water-Insoluble Precipitates of Type 2 Serum

(All mice received injections of 0.5 c c. of culture diluted 1:100)

	Dilution of Sample					
	1:5	1:10	1:20	1:40	1:80	1:160
Precipitate from 20 c c. serum made up to 20 c c. (dilution 1:8)	S S	S S	S S	S S	S S	36 S
Part of dissolved precipitate 54 C. for 30 min.	S S	S S	S S	S S	60 S	36 S
Portion precipitated with 5 mg. soluble specific substance	132 36	36 36	36 44	36 36	36 36	18 18
Same as above, 54 C. for 30 min.	S S	36 72	36 44	20 24	20 24	36 36
Control serum	S S	S S	S S	S S	S S	36 S
	1:10,000,000			1:1,000,000		
Organism control	36 36			36 36		

Numbers refer to hours of survival.

From the foregoing experiments, it is obvious that a union of protective substance with the soluble specific substance produces a combination which does not protect; in other words, the protective antibody is neutralized by the soluble specific substance with the formation of a compound found to be relatively insoluble in water and neutral salts. Whether or not it is true that pure soluble specific substance possesses an action antagonistic to defense, the samples used in these investigations displayed this characteristic. Consequently, to obtain the protective antibody free from the precipitating agent, not only must there be a dissociation, but also a separation of dissociated

[9] Felton, L. D.: Boston Med. & Surg. Jour., 1924, 190, p. 819.

members. The first part of this process has been utilized by Huntoon,[10] who dissociated the substance precipitated from serum by living organisms by heating the suspension in water, neutral salts, saccharose, sodium carbonate, and many other reagents. An analysis of antibody prepared by him and also that prepared according to his technic, together with a dissociated precipitate obtained with the soluble specific substance, showed the presence of a substance which caused a specific precipitation in immune serum; in other words, the precipitinogen is liberated by heat and consequently free to react with more antibody. Experiments on dissociation of precipitates with the soluble specific substance are to be given in a subsequent report.

NEUTRALIZATION IN VIVO OF ANTIBODY SOLUTION BY SOLUBLE SPECIFIC SUBSTANCE

Exper. 5.—In preliminary experiments it was found that, by the injection of soluble specific substance into mice simultaneously with immune serum and virulent pneumococci, the serum failed to protect.

TABLE 6

NEUTRALIZATION IN VIVO OF ANTIBODY SOLUTION BY SOLUBLE SPECIFIC SUBSTANCE

Dose of Soluble Specific Substance, Mg.	Dilution of Antibody					
	1:5	1:10	1:20	1:40	1:80	1:160
5.0	S S	21 S	18 18	21 25	18 36	18 24
0.5	S S	S S	20 72	18 44	18 18	18 18
0.05	S S	S S	S S	S S	20 60	46 S
0.005	S S	S S	S S	S S	60 S	60 108
Control	S S	S S	S S	S S	S S	36 S
		1:10,000,000			1:1,000,000	
Organism control		38 38			20 20	

Numbers refer to hours of survival.

In a more comprehensive test, a sample of antibody free from antagonistic action in a dilution of 1 : 5 was used in a series of dilutions of 1 : 5 to 1 : 160. As may be seen from table 6, mice received injections in duplicate with the various dilutions of antibody and 0.5 c c. of 1 : 100

[10] Jour. Immunol., 1921, 6, pp. 117, 123, 185.

virulent culture of type 2 pneumococcus. Each dilution was injected from separate syringes in the following order: soluble specific substance, organisms and antibody solution. The results of the experiment show definitely that with this sample of antibody there was a neutralization of the protective action approximately in proportion to the amount of the soluble specific substance injected.

A repetition of this experiment (table 7) was made with an antibody solution of higher value. Not considering the 1 : 5 dilution which had an antagonistic effect, the results here agree well with the preceding; for it is seen that with a concentration of 1 : 100 of the specific substance, relatively the same amount of protective material was neutralized.

TABLE 7

Neutralization in Vivo of Antibody Solution by Soluble Specific Substance

Dose of Soluble Specific Substance, Mg.	Dilution of Antibody							
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
5.0	24	S	S	18	18	18	18	18
	36	S	S	S	20	18	18	18
0.5	18	S	S	S	S	S	48	18
	18	S	S	S	S	S	36	44
Control	18	S	S	S	S	S	S	36
	36	S	S	S	S	S	S	48
		1:10,000,000				1:1,000,000		
Organism control		36				36		
		36				36		

Numbers refer to hours of survival.

The results recorded in this report as to in vitro and in vivo neutralization of the protective properties of serum and antibody solutions by the soluble specific substance of type 2 pneumococcus are in accord with the work of Cole,[4] who demonstrated the neutralization of antipneumococcus immune bodies by infected exudates and serums. This investigator observed that both in empyema fluids and in the blood of rabbits infected with pneumococci, there are present large amounts of soluble substances which have the property of neutralizing pneumococcus antibodies.

SUBSTANCES SEPARATED FROM IMMUNE SERUM AND ANTIBODY WHICH CAUSE PRECIPITATION

Exper. 6.—This experiment represents an endeavor to separate from immune serum a soluble specific substance. It was undertaken in view of the presence of a specific antagonistic substance in immune

serum, which is demonstrated by the fact that in the protection tests mice receiving injections with large amounts of serum or antibody solution do not survive multiple lethal doses of pneumococci.[2] The following is a brief description of the methods used and results of this study. In table 8 are shown the precipitating properties of the final products, the numbers referring to the material and methods.

A. The precipitate from 40 liters of type 1 antipneumococcus serum prepared by dilution of the serum with an equal volume of

TABLE 8

SUBSTANCES SEPARATED FROM IMMUNE SERUM AND ANTIBODY WHICH CAUSE PRECIPITATION

Description	Serums, Type	Dilutions of Samples						
		1:4	1:8	1:16	1:32	1:64	1:128	1:256
A								
Type 1 serum, F69 precipitate from 40,000 c c., 1:1 dilution leached out with NH_4OH	1	+	+	+	+	+	±	—
	2	+	+	+	+	±	—	—
	3	+	+	+	+	±	—	—
	Normal	+	+	±	—	—	—	—
B								
Type 1 antibody, lot No. 34 supernatant from heat coagulation	1	±	—	—	—	—	—	—
	2	+	+	+	±	—	—	—
	3	+	+	+	±	—	—	—
	Normal	—	—	—	—	—	—	—
C								
Type 1 antibody, lot No. 34 coagulum from above digested with pepsin	1	+	+	+	+	—	—	—
	2	+	+	+	+	—	—	—
	3	+	+	+	+	±	—	—
	Normal	+	—	—	—	—	—	—
D								
Type 2 serum, 1312 precipitate from 1,000 c c., 1:1 dilution leached out with NH_4OH	1	±	—	—	—	—	—	—
	2	+	+	+	±	—	—	—
	3	±	—	—	—	—	—	—
	Normal	—	—	—	—	—	—	—
E								
Type 2 serum, 1312 precipitate from 1,000 c c., coagulated with heat and leached out with NH_4OH	1	±	—	—	—	—	—	—
	2	+	+	+	±	—	—	—
	3	+	+	+	±	—	—	—
	Normal	—	—	—	—	—	—	—

water was leached out on the water bath 12 times with normal ammonium hydroxide. The leachings were combined, neutralized with acetic acid, filtered, dialyzed, and again filtered. The filtrate was evaporated to dryness 5 times, each time taken up in distilled water, and the insoluble precipitate separated by centrifugalization. The final residue was taken up in 10 c c. of 0.85% sodium chloride and tested as shown in the table. It will be noted that the solution so prepared caused a precipitation in the 3 types of antipneumococcus serums and to a less degree in normal serum. These results would indicate that the precipitating substance is similar to the nonspecific protein obtained from the pneumococcus by

Avery and Heidelberger;[11] for they noted that such protein causes precipitation in all 3 types in about the same degree.

B and *C*. These letters represent an experiment which was performed on a type 1 antibody solution which for some unknown reason had become nonprotective. One and one-half liters of this material was coagulated by heat. *B* in the table represents the supernatant, and *C* the coagulum from the same, which was digested almost entirely free of protein by pepsin. The supernatant was evaporated to dryness several times, centrifugalized, dialyzed, and again treated as in the last part of exper. *A*. Dialysis was carried out over a long period with the pepsin digest in order to reduce the dialyzable substances to a minimum. The residue was also worked up in the same manner as *A*. The results with these are almost similar to the ones with the type 1 precipitate, and show that the precipitating substance causes a nonspecific precipitation in antipneumococcus serum.

D. One liter of type 2 serum was treated by the same technic as was used with *A*. In this case, however, the final product gave a precipitate essentially specific to type; at least, the precipitation extended to a greater dilution with the type 2 serum.

E. The precipitate obtained by diluting 1 liter of type 2 serum with an equal volume of water was leached out. The general procedure followed was that outlined in *A*. Again it is seen that the final material caused a precipitation in all types of serums, but greater in types 2 and 3.

SUMMARY AND DISCUSSION

The soluble specific substance of type 2 pneumococcus, prepared according to a method[3] which eliminates all except a minimum amount of nitrogen, was injected into mice in extremely small doses, together with attenuated type 2 pneumococci. The results indicate an antagonistic effect on the defense of the animals, or an actual increase in the virulence of the micro-organisms from ten to one hundred fold. The injection of the specific substance alone into mice in doses many times greater than used with the attenuated cultures of pneumococci demonstrated that the lots of specific substance used were in themselves nontoxic.

Experiments are recorded which show that the protective substance of antipneumococcus serum and antibody solutions are neutralized both in vitro and in vivo, and indicate that the neutralization is approximately in proportion to the amount of soluble specific substance present. Furthermore, the results indicate that the soluble specific substance

[11] Jour. Exper. Med., 1923, 38, p. 81.

must be considered at least as one probable source of the antagonistic effect commonly noted in protection tests with the larger amounts of antipneumococcus serums and antibody solutions. Indeed these serums and solutions, when treated even with very low concentrations of the specific substance, exhibit to a marked degree the common zonal phenomenon in regard to protection, which appears to be entirely analogous to that obtained in certain instances with untreated antipneumococcus serums and antibody solutions. This analogy appears to have a certain degree of confirmation in the experiments here recorded in which it was shown that precipitable "residues" may be extracted from such serums and solutions.

The results obtained in this study as to in vitro and in vivo neutralization of the protective properties of serum and antibody solution by the soluble specific substance are in accord in certain respects with those of Cole,[4] who noted the neutralization of antipneumococcus immune bodies by infected exudates and serums containing large amounts of "soluble substances." Indeed, Cole suggested that his results offer an explanation for the fact that infections in the partially immunized animal tend to remain localized, while a definite septicemia results only after the soluble substances have accumulated in the blood serum in sufficient amounts to neutralize the specific antibodies. For this reason, early and intensive treatment of pneumonia is recommended by him, especially that resulting from infection with types 2 and 3 pneumococci, which appear to be able to liberate soluble specific substances in greatest amount. Bail[5] has given to such substances the name "agressins," and has suggested that they are secreted by the microorganisms during their growth in the animal body. According to this investigator, the virulence and invasive power of bacteria depend on their ability to secrete these substances. Wassermann and Citron[6] believed, however, that the so-called "agressins" represent merely bacterial substances which go into solution in the animal body, or during growth or autolysis in vitro, and may fix immune bodies in the same manner as dead bacteria. Studies by Hoke,[12] Rosenow,[13] Tschistowich and Jurewich,[14] Zade,[15] and Nunokawa[16] have demonstrated the part such substances play in experimental pneumococcus infection, especially in regard to their effect on phagocytosis. It would appear that the

[12] Wien. klin. Wchnschr., 1905, 18, p. 348.
[13] Jour. Infect. Dis., 1907, 4, p. 285.
[14] Ann. de l'Inst. Pasteur, 1908, 22, p. 611.
[15] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1909, 2, p. 81.
[16] Ibid., 3, p. 172.

soluble specific substance of type 2 pneumococcus used in the present investigation, assuming that the results are due to an actual increase in virulence of the attenuated culture of this organism, produced an effect more or less similar to that reported by Rosenow in case of the so-called "virulin." However, a great deal more experimental work must be carried out, using a soluble specific substance of unquestionable purity, before it might be possible to suggest an identity or even a relationship between the latter substance and the hypothetical one just mentioned. We wish to defer making final conclusions as to the biologic significance of the soluble specific substances of pneumococci until further investigations, now being carried on, have been completed.

THE SPECIFIC PRECIPITATES OBTAINED FROM ANTIPNEUMOCOCCUS SERUM AND ANTIBODY SOLUTION BY THE SOLUBLE SPECIFIC SUBSTANCES OF PNEUMOCOCCI *

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In the preceding report,[1] it was shown that the soluble specific substances isolated from pneumococci cause a precipitation and neutralization of the protective substance of antipneumococcus serums and antibody solutions. In the case of type 2 serum, a concentration of 1:2,000 to 1:15,000 of the specific substance was observed to cause complete precipitation. This is also the concentration of soluble specific substance reported by Morgan[2] to produce maximum precipitation. With higher concentrations there is a prozone of partial precipitation; also with lower concentrations the precipitation is incomplete, due to an insufficient amount of the specific precipitating material. These observation are in accord with those of Gay and Chickering,[3] who have reported precipitation of practically all the protective substance in antipneumococcus serum by an antigen consisting of dissolved pneumococci. Similarly, Huntoon[4] has precipitated in part the protective substance from antipneumococcus serum by use of the living organisms.

The historical development of the dissociation of antibody-antigen complexes has been given by Chickering,[5] Huntoon,[4] Berger,[6] Zinsser,[7] and others, and it will be profitable here to review only some of the most important investigations in which the principles involved or methods used have a direct bearing on the dissociation of the specific precipitates of antipneumococcus serum. The methods follow the principles elucidated by the fundamental researches of Hahn and Trommsdorf,[8] Landsteiner and Jagic,[9] Morgenroth,[10] Pfeiffer and Friedberger,[11] Von Liebermann and Fenyvessy,[12] Bail and Tsuda,[13] Spaet,[14] Kosaki[15] and others. It was early recognized that antibody would combine with its homologous

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1 Felton and Bailey: Jour. Infect. Dis., 1926, 38, p. 131.
2 Jour. Immunol., 1923, 8, p. 449.
3 Jour. Exper. Med., 1915, 21, p. 389.
4 Jour. Immunol., 1921, 6, p. 117.
5 Jour. Exper. Med., 1915, 22, p. 248.
6 Berl. klin. Wchnschr., 1923, p. 1176.
7 Infection and Resistance, 1924, p. 315.
8 München. med. Wchnschr., 1900, 47, p. 413.
9 Ibid., 1903, 50, p. 764.
10 Ibid., p. 61.
11 Centralbl. f. Bakteriol. I. O., 1903, 34, p. 70.
12 Ibid., 1908, 47, p. 274.
13 Ztschr. f. Immunitätsforsch. u. exper. Therap., 1909, 1, p. 546.
14 Ibid., 1910, 7, p. 712.
15 Jour. Immunol., 1918, 3, p. 109.

antigen, and in the case of formed antigens such a combination could be washed free from most of the other serum constituents. These investigators noted the reversibility of the antigen-antibody combination by means of appropriate physical or chemical agents, such as heat, dilute acids or alkalis, certain salts, solutions of saccharose, etc. By indirect adsorption methods, Gay,[16] Muir and Martin,[17] and Zinsser[18] found that when a serum containing the precipitin antibody is treated with a proper precipitinogen, the resulting precipitate carried down with it a large proportion of all the antibodies of the serum. About this time, Krauss[19] and Matsui[20] demonstrated the dissociation of antigen and antibody, both in vivo and in vitro.

The first application of the known principles for the purification of pneumococcus antibodies was made by Gay and Chickering,[3] who studied the precipitate formed in immune serum by the addition of a water clear extract of pneumococci. The precipitate was found to contain practically all the protective antibodies of the original serum and relatively small amounts of protein; and when washed and suspended in salt solution to the original volume of serum, it protected as well as the whole serum. Solutions of the precipitates were not necessary to insure protection. This investigation was extended by Chickering,[5] who found that the specific precipitates obtained from antipneumococcus serum by the addition of either pneumococcus extracts or the living organisms may be dissociated by suitable chemical or physical agents, such as normal salt solution with dilute sodium carbonate at 42 C. The resulting clear supernatant contained about one-fiftieth of the serum protein, and when made to the original volume, protected almost as well as the whole serum. Agglutinins and precipitins were present, as well as protective substances. It is evident, however, that the final product also contains considerable amounts of pneumococcus antigen, since it was found to stimulate an active immunity in experimental animals.

The most recent contribution to this problem has been made by Huntoon.[4] He confirmed and extended the observation of Chickering, using live washed cultures instead of dissolved pneumococci as a precipitating agent. A successful dissociation of antibody and pneumococci was reported by him. The agglutinated organisms were suspended in any one of several solutions and heated. Best results were obtained with 0.5% sodium carbonate in salt solution. This antibody solution, having only small amounts of protein, contains in addition to the antibodies a considerable amount of bacterial material, possibly in the form of the "residue antigens" described by Zinsser and Parker.[21]

In making a comparison of these three precipitating agents, namely, dissolved pneumococci, living pneumococci, and soluble specific substance of pneumococci prepared by the method of Heidelberger and Avery,[22] it is evident that the first and second are the most toxic. However, the last apparently has an agressin-like property, as shown in a preceding report. It is thus seen that in the dissociation of the material precipitated by any of these precipitating agents, the problem is twofold, namely, to bring about complete dissociation, and to separate the products of dissociation. Consequently, the study here reported includes attempts to dissociate the precipitate obtained by the soluble specific substance and to separate the dissociated components.

[16] Centralbl. f. Bakteriol. I. O., 1905, 39, p. 603; Univ. Calif. Pub. Path., 1911, 2, p. 23.
[17] Jour. Hyg., 1906, 6, p. 265.
[18] Jour. Exper. Med., 1912, 15, p. 529.
[19] Biochem. Ztschr., 1913, 56, p. 457.
[20] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1914, 23, p. 233.
[21] Jour. Exper. Med., 1923, 37, p. 275.
[22] Ibid., 1924, 40, p. 301.

This investigation is limited to type 2 soluble specific substance. Protective value was estimated in all cases against 0.5 c c. of an 8-hour broth culture diluted 1 : 100. This dose of organism was employed to assure more definite end-points; for it has been found that irregular results follow the use of a larger number of organisms with types 2 and 3 immune serums or antibody solutions.

In the main, the following subjects have been taken up: (1) the dissociability of the precipitate; (2) separation of the dissociated components; (3) study of hydrogen ion concentration of minimum solubility of the specific precipitates and its effect on dissociation; (4) the combining ratio by weight of the soluble specific substance with the protective precipitate.

The amount of dissociation for the most part has been measured by the following methods: nitrogen content, protection tests and immunologic tests in vitro (agglutination and precipitin reactions), the dissociated product being used first as antibody against a known precipitinogen, and second as precipitinogen against a known antibody.

DISSOCIABILITY BY HEAT OF THE SPECIFIC PRECIPITATE IN WATER AND HYDROCHLORIC ACID

Exper. 1.—This experiment is a study of the dissociability of the specific precipitate. A sample of this precipitate prepared by precipitation of a potent type 2 antipneumococcus serum with 1 : 5,000 concentration of soluble specific substance was washed first with a small quantity of 0.85% sodium chloride and again, after collecting the supernatant, with an equal volume of water. After centrifugalization the precipitate was suspended in distilled water and divided into 3 portions. One was dissolved in N/100 hydrochloric acid; another was suspended in 10% saccharose solution and a third was dissolved in N/20 hydrochloric acid. The 3 fractions were made to equal volumes and along with the 2 washings mentioned above were heated at 54 C. for one-half hour. To the sample dissolved in N/20 hydrochloric acid, sodium chloride was added to 25% saturation, and the precipitate thus obtained made to the original volume with distilled water. Since the precipitate was not entirely soluble, the insoluble portion was suspended in the solution and the mixture used in protection tests. From table 1 it is seen that the two washings contained a relatively large amount of active protective substance—0.009 gm. of nitrogen in the case of the sodium chloride washings protecting against 0.01 c c. of a culture of virulent pneumococci. The sample of precipitate dissolved in N/100 hydro-

chloric acid, although having almost twice the amount of nitrogen per unit volume, exhibited little protective value. On the other hand, the sample suspended in 10% saccharose was approximately as protective as the washings. The dissolved precipitate obtained from the N/20 hydrochloric acid solution by 25% saturation with sodium chloride displayed greater protective action than the N/100 hydrochloric acid solution, whereas the supenatant from this precipitate, of low protein content, not only failed to protect, but even hastened the death of the animals injected. It will be noted also that in the case of the precipitate with sodium chloride duplicate mice died in the dilution of 1 : 5, while all survived in dilutions of 1 : 10 to 1 : 40.

TABLE 1

DISSOCIABILITY BY HEAT OF THE SPECIFIC PRECIPITATE IN WATER AND HYDROCHLORIC ACID

All mice received injections of 0.5 c c. of an 8-hour broth culture diluted 1:100

Reagents	Nitrogen, Mg./C c.	Nitrogen in Protective Dose	Dilutions of Samples					
			1:5	1:10	1:20	1:40	1:80	1:160
0.85% NaCl washing	1.442	0.009	60 S	S S	S S	S S	S S	S S
Water washing	1.680	0.021	S S	S S	S S	S S	S S	72 S
N/100 HCl	2.604	0.065	60 S	S S	60 60	84 S	36 48	60 48
10% saccharose	2.044	0.026	S S	S S	S S	S S	S S	36 44
N/20 HCl precipitated with 25% saturation of NaCl	1.260	0.021	24 36	S S	S S	S S	36 S	36 48
Supernatant from NaCl precipitate	0.098	.....	18 18	18 22	20 22	18 20		
			1:10,000,000			1:1,000,000		
Organism control			36 36			36 36		

S indicates survival; numbers refer to hours of survival.

DISSOCIABILITY AFTER REPEATED WASHING WITH 3% SODIUM CHLORIDE

Exper. 2.—This experiment deals with the comparative dissociation of heated and unheated samples of specific precipitate after washing with 3% sodium chloride. The precipitate was prepared by the addition of a 1 : 2,500 dilution of specific substance in 100 c c. of 0.85% sodium chloride to 100 c c. of a potent type 2 serum, and the mixture incubated for 2 hours at 37 C. The precipitate was then washed 4 times with 5 c c. of 3% sodium chloride shaking in a machine for

1½ hours each time. This process was repeated the following day with the same concentrations and volumes except that the shaking was continued by hand for one-half hour periods. Heated and unheated suspensions of the precipitate in 3% sodium chloride, their respective supernatants, the supernatant serum after precipitation with soluble specific substance, and a sample of the original serum were tested for protection as indicated in table 2. It may be seen that the first and second lots of washings with 3% sodium chloride gave little protection, although

TABLE 2

DISSOCIABILITY AFTER REPEATED WASHING WITH 3% SODIUM CHLORIDE

All mice received injections of 0.5 c.c. of an 8-hour broth culture diluted 1:100

Samples	Dilutions of Samples						
	1:5	1:10	1:20	1:40	1:80	1:160	1:320
First to fourth washing with 3% NaCl, combined	36 36	36 36	36 36	36 36	20 21	36 60	20 36
Fifth to eighth washing with 3% NaCl, combined	84 84	S S	60 60	36 36	36 36	36 36	20 36
Unheated suspension in 3% NaCl.....	36 36	36 S	S S	36 60	60 96		
Suspension heated 55 C. for 30 min.	36 36	S S	S S	S S	S S	36 S	36 36
Supernatant of unheated suspension....	S S	18 84	60 S	60 84	18 21	21 36	
Supernatant of heated suspension.....	S S	S S	36 S	60 S	36 36	60 60	
Serum (1:1) after precipitation with soluble specific substance	60 S	36 36	36 36	18 36			
Serum control	S S	S S	S S	S S	S S	60 S	
Organism control.....		1:10,000,000 36 36			1:1,000,000 24 36		

S indicates survival; numbers refer to hours of survival.

judging from other experiments they contained considerable protein, whereas the suspension heated at 54 C. showed a high protective titer. However, when this heated suspension was freed from precipitate, the supernatant protected only in a dilution of 1 : 10. On the other hand, the unheated suspension exhibited no protection, or at least a zonal reaction, even though its supernatant when heated protected to a dilution of 1 : 5. The lack of protection in the supernatant of serum precipitated at a concentration of 1 : 5,000 as a final dilution of the specific substance shows that practically all the protective material had been removed.

From this experiment 3 inferences may be drawn: (1) The precipitate containing the protective antibody is relatively insoluble in 3% sodium chloride. (2) The material insoluble in sodium chloride when injected into mice acts only to a slight degree as a protective agent. This is perhaps due in part to dissociation before the substance is injected and also to dissociation within the animal. In the latter case, the rate of dissociation is so slow that the amount of soluble specific substance released is insufficient to paralyze the defense of the animal. (3) The amount of soluble specific substance necessary to neutralize the protective antibody is small compared with the dose which displays to a marked degree a paralyzing effect on the animal's defense.

EFFECT OF HEAT ON DISSOCIATION WITH VARIOUS REAGENTS

Exper. 3.—The reagents used in this study of dissociation were the common ones tried by Huntoon [4] in his work on a protective antibody from antipneumococcus serum. A precipitate obtained in the usual

TABLE 3

EFFECT OF HEAT ON DISSOCIATION WITH VARIOUS REAGENTS

Reagents	Protective Titer	Antagonistic Titer	Nitrogen Mg./C c.	Nitrogen in Protective Dose	Precipitinogen	Precipitins (Supernatants as Antibody)
N/100 HCl	0	....	2.738	.....	1:256	1:40
N/100 H_2SO_4	0	....	2.741	.....	.....	1:80
N/100 NaOH	20:160	2:10	1.610	0.1000	1:512	1:40
N/100 Na_2CO_3	10:640	2:5	0.679	0.0100	1:2	1:40
10% saccharose	2:80	0	1.740	0.0218		
N/100 HCl and 10% saccharose	2:20	0	0.249	0.1245	.....	1:80
N/100 H_2SO_4 and 10% saccharose	5:40	1:2	0.245	0.6125	.....	1:80
N/100 NaOH and 10% saccharose	10:100	1:5	0.630	0.0400	.....	1:20

manner was washed once with 0.85% sodium chloride and suspended in a small amount of water. From this suspension were taken 8 equal parts. Sufficient of the various reagents shown in table 3 were added to the different portions to make the desired concentrations, and the samples made to the same volume with water. The tubes were then heated at 55 C. for 30 minutes, shaking occasionally. The precipitates were thrown down in a centrifuge and the supernatants used for the various tests. As seen in table 3, from the standpoint of minimum nitrogen in a protective dose, N/100 sodium carbonate is superior to any of the other reagents. This fact is in accord with the observations of Huntoon, who showed that 0.5% sodium carbonate apparently makes the best medium for dissociation of his precipitate. In this experiment

saccharose was found to be next best, whereas the hydrochloric acid and sulphuric acid solutions showed no protection. The nitrogen content of the supernatants of these samples was, however, higher than that of any of the others. It will be observed from the table that the supernatants, whether they were protective or not, contained protein which was precipitated by the specific substance. Furthermore, the hydrochloric acid, the sodium hydroxide, and the sodium carbonate solutions contained antibodies, as evidenced by the precipitin tests, all giving positive reactions in dilution 1 : 40. When these samples were used as precipitinogens against a known antibody solution, it was seen that the hydrochloric acid and the sodium hydroxide samples gave a relatively high titer, respectively, 1 : 256 and 1 : 512, whereas the carbonate sample was positive only in a dilution of 1 : 2. It will be noted in the column showing protective titer in the table that there are two sets of figures (e. g., 20-160). The first set of figures shows the dilution giving antagonistic action (death in mice), and the second the dilution protecting against a unit dose of organisms. In this connection, it is seen that there is an apparent relationship between the amount of antagonistic substance present and the precipitating value of the samples.

FRACTION OF PRECIPITATE DISSOCIATED BY HEAT, INSOLUBLE IN 18% SODIUM SULPHATE

Exper. 4.—The antagonistic effect, seemingly due to the presence of the soluble specific substance as stated in an introductory paragraph, is well brought out in the previous experiment. Since the specific substance is soluble in 18% sodium sulphate, and the protective antibody is insoluble, attempts were made to separate the protective substance from the soluble specific substances by this neutral salt. In the same manner as in the preceding experiment, dissociation was produced by means of these reagents, namely, hydrochloric acid, sodium hydroxide, and sodium carbonate, each combined with saccharose. After heating and centrifugalization, sodium sulphate to make 18% was added to the clear supernatant. The resulting precipitates were taken up in equal volumes of 1% sodium chloride. The precipitates were not entirely soluble, so that the mixture of suspension and solution was used to estimate the nitrogen and protective titer. The results given in table 4 show the possibility of separating the protective substance from most of the soluble specific substance by a single precipitation with sodium sulphate. It will be noted, however, that antagonistic action is present even by the use of this method in a dillution of 1 : 2. If the amount of nitrogen for a protective dose is considered, the best results are found

in the case of sodium carbonate with saccharose, the material so obtained being of high protective value. It should be pointed out that the different samples contained a relatively small amount of substance precipitable on addition of the soluble specific substance.

TABLE 4

FRACTION OF PRECIPITATE DISSOCIATED BY HEAT, INSOLUBLE IN 18% SODIUM SULPHATE

Reagents	Protective Titer	Nitrogen, Mg./C c.	Nitrogen in Protective Titer	Precipitins (Supernatants as Antibody)
N/100 HCl and 10% saccharose....	0	0.316	0	1:10
N/100 NaOH and 10% saccharose..	2:40	0.506	0 1260	1:5
N/100 Na_2CO_3 and 10% saccharose	2:160	0.406	0.0250	1:5

EFFECT OF ALKALI ON DISSOCIATION

Exper. 5.—The successful experiments with sodium carbonate suggest the possibility of a high degree of dissociation on the alkaline side without resorting to the heating process. To test this assumption heated and unheated samples with sodium carbonate and with disodium phosphate were run in parallel. A sample of precipitate obtained in the usual manner and washed once with 0.85% sodium chloride was used. the results, given in table 5 show that in all the experiments the mice receiving injections with the undiluted supernatants failed to survive.

TABLE 5

EFFECT OF ALKALI ON DISSOCIATION

Reagents	Protective Titer	Nitrogen, Mg./C c.	Nitrogen in Protective Dose	Precipitins (Supernatants as Antibody)
N/100 Na_2CO_3	2:50	2.387	0.477	0
N/100 Na_2CO_3 heated at 55 C. for 30 min.	2:200	2.737	0.137	1:20
N/100 Na_2HPO_4	2:80	0.182	0.025	0
N/100 Na_2HPO_4 heated 55 C. for 30 min.	2:80	0.420	0.052	0
N/100 Na_2CO_3 heated fraction insoluble in 18% Na_2SO_4.....	2:400	0.710	0.018	1:40
NaCl washing from insoluble fraction of Na_2SO_4 precipitate	2:40	0.168	0.042	0

It will be noted that in the heated sodium carbonate and disodium phosphate samples there was an increase in dissociation as indicated by the increase in nitrogen content of the supernatants of these samples. However, there was an increase in the protective titer in the sample heated with carbonate, with apparently no difference in titer between the heated and the unheated phosphates.

In addition in this experiment, a large sample of precipitate was dissociated with the carbonate by heat and the supernatant precipitated with 18% sodium sulphate. The precipitate so obtained was taken up with water (sufficient sodium sulphate being present to dissolve the salt-soluble antibody) to make the original volume and the insoluble portion separated by centrifugalization. The supernatant was used in tests for protection and nitrogen content. It may be seen (table 5) that this sample gave per milligram of nitrogen higher protection than parallel solutions not so precipitated. Furthermore, the fraction insoluble in neutral salts washed 3 times with small amounts of 1% sodium chloride and made to volume, contained one-fourth as much protein as the preceding sample, yet the protective value was only one-tenth as great.

PRECIPITATION OF PROTECTIVE SUBSTANCE FROM HYDROCHLORIC ACID SOLUTION BY SODIUM CHLORIDE

Exper. 6.—The preliminary experiment, given in table 1, revealed the possibility of precipitating the protective antibody from a hydrochloric acid solution by high concentrations of sodium chloride. Several experiments were carried out to determine the effects of various concentrations of this acid on the protective antibody. These experiments were made both at room temperature and at icebox temperature (8 C.). Concentrations of N/2, N/4, N/8, N/16, N/20, and N/100 hydrochloric acid were used to dissolve samples of the precipitate. After standing for from 4 to 24 hours, these were tested for protection and dissociation of the soluble specific substance. In all acid solutions the specific substance was dissociated in great degree, but a concentration greater than N/20 hydrochloric acid caused a rapid destruction of the protective antibody.

In carrying out the present experiment a similar precipitate was dissolved in N/20 hydrochloric acid, divided into 3 equal portions, and sodium chloride added to make respectively a 25%, a 50%, and a saturated solution. The resulting precipitates were collected by means of the centrifuge, and sufficient water added to make each the original volume. The incomplete solubility of these precipitates in neutral salts would indicate that there had not been complete dissociation at this concentration of hydrochloric acid. A study of the results given in table 6 shows that in the precipitate from the saturated solution there was a substance antagonistic to the defense as high as a dilution of 1 : 20, while protection was exhibited from this point to a dilution of

1 : 80. It would thus appear that all the dissociated protective antibody, and a considerable amount at least of the soluble specific substance, are precipitated from a solution saturated with sodium chloride. The results with 50% saturation of this salt are somewhat confusing. For, although no demonstrable antagonistic substance is precipitated at this concentration, there is a low yield of protective material. In the case of 25% saturation there is also no measurable antagonistic effect, but, on the other hand, a highly protective solution. It will be noted here again that in the suspension control the mice receiving injections with the 1 : 5 and 1 : 10 dilutions died, while those with the 1 : 20 dilution survived.

TABLE 6

Precipitation of Protective Substance from Hydrochloric Acid Solution by Sodium Chloride

All mice received injections of 0.5 c c. of an 8-hour broth culture diluted 1:100

Percentage Saturated NaCl	Dilutions of Samples					
	1:5	1:10	1:20	1:40	1:80	1:160
Saturated	20 24	24 36	48 S	S S	S S	36 36
50% saturated	S S	S S	S 48	36 60	60 36	48 84
25% saturated	S S	S S	S S	S S	S S	84 S
Suspension control	20 22	36 96	S S			
		1:10,000,000			1:1,000,000	
Organism control		36 S			36 36	

S indicates survival; numbers refer to hours of survival.

The precipitation of the hydrochloric acid solution by 25% saturation with sodium chloride has been repeated several times, with variable results. Evidently N/20 hydrochloric acid causes an inherent change of the protein which makes it less soluble in neutral salts, or, in other words, causes denaturization. This is the common experience when proteins are salted out from very acid solutions, as emphasized by the work of Chick and Martin.[23] However, it is possible that with lower concentrations of acids and higher concentrations of neutral salts a more satisfactory separation could be made.

[23] Jour. Physiol., 1911, 43, p. 1; 1912, 45, p. 261.

EFFECT OF REPRECIPITATION OF A HYDROCHLORIC ACID SOLUTION OF SPECIFIC PRECIPITATE WITH SODIUM HYDROXIDE

Exper. 7.—The object of this experiment was to measure the dissociation of the precipitate as influenced by repeated precipitations from

TABLE 7

EFFECT OF REPRECIPITATION OF A HYDROCHLORIC ACID SOLUTION OF SPECIFIC PRECIPITATE WITH SODIUM HYDROXIDE

All mice received injections of 0.5 c c. of an 8-hour broth culture diluted 1:100

Supernatants	Nitrogen, Mg./C c.	Protection Tests: Dilution of Culture 1:100	1:1,000	1:10,000	Precipitinogen
1	0.403	S S	S S	S S	1:2
2	0.109	84 S	60 60	S S	1:4
3	0.036	24 36	60 60	S S	1:16
4	0.042	18 20	44 60	44 S	1:32
5	0.025	36 36	36 S	108 S	1:16
6	0.078	36 36	S S	36 S	1:16
7	0.123	36 36	S S	108 S	1:8
8	0.039	24 36	S S	S S	1:8
9	0.031	20 36	20 60	S S	1:4
10	0.028	36 36	36 44	S S	1:4

		Dilutions of Sample 1:5	1:10	1:20	1:40	1:80	1:160	1:320
Final precipitate	3.010	20 24	36 108	36 56	24 24	24 36	18 20	18 18
Original precipitate	.....	24 24	36 24	S S	S S	60 S		

	1:10,000,000	1:1,000,000
Organism control	36 36	36 36

S indicates survival; numbers refer to hours of survival.

acid solution by neutralization with sodium hydroxide, and to correlate this dissociation with protection. The procedure was as follows: The precipitate from 125 c c. of serum was dissolved in 15 c c. of N/20 hydrochloric acid and then neutralized to approximately P_H 6.4 to

P_H 6.6 with N/20 sodium hydroxide; the resulting precipitate after centrifugalization was then redissolved in N/20 hydrochloric acid and reprecipitated with N/20 sodium hydroxide. This process of solution and reprecipitation was repeated 10 times. The supernatants were tested for protective power and used as precipitinogens for estimation of the presence of free soluble specific substance. As may be seen from table 7, four points should be emphasized: (1) Very little protein was lost as indicated by the nitrogen content of the various supernatants. (2) The amount of dissociation varied, the dilution giving a precipitate with type 2 serum, reaching a maximum in the fourth sample. (3) The degree of protection of the supernatants varied inversely with the amount of dissociated specific substance. (4) The final sample containing as much as 3 mg. of nitrogen per c c. had no measurable protective action, whereas the original precipitate protected in dilutions of 1 : 20 to 1 : 80.

The reverse of this experiment, that is, dissolving the precipitate first in N/20 sodium hydroxide and neutralizing with N/20 hydrochloric acid gave about the same results throughout, with the exception that the final precipitate had lost only 50% of its protective value. In addition to these, other experiments were carried out in the same manner, with the result that the protective titer suffered only a small loss after the third and fourth reprecipitations.

THE PRESENCE OF ANTIBODY IN THE PRODUCTS OF DISSOCIATION

Exper. 8.—Although a comprehensive study has not been made of the immunologic characteristics of the dissociated precipitate, in all the samples so far tested the product of dissociation soluble in neutral salts or dilute acid and alkali contains agglutinins and precipitins. This experiment was planned to show the correlation between protection, nitrogen content, and precipitin titer of samples of precipitate dissociated with N/100 sodium carbonate, 10% saccharose, and 10% saccharose in 9% sodium chloride. This concentration of sodium chloride was used through error, but other experiments have shown that from 1 to 3% are equally effective, the percentage to be used depending on the concentration of the suspended precipitate. The results of the experiment (table 8) show that with the 3 samples the amount of nitrogen, as an index of the protein present, which protects against a million lethal doses of the organisms is quite constant; that is, with the saccharose sample there was a small amount of protein in solution, and

protection extended proportionately only to a low dilution of the supernatant. Precipitin tests carried out by using dilutions of the supernatants of the 3 samples as antibody against soluble specific material gave the same titer in the carbonate and also the saccharose-sodium chloride medium, but were negative in the saccharose sample. When the supernatants were used as precipitinogens, positive reactions occurred only in the carbonate and saccharose-sodium chloride samples, the latter giving the higher titer. This fact is of interest as the 1 : 5 dilution in this case failed to protect. A possible relationship is thus shown between the amount of precipitinogen present and the agressin-like phenomena described elsewhere.[1]

TABLE 8

THE PRESENCE OF ANTIBODY IN THE PRODUCTS OF DISSOCIATION

Description	Nitrogen, Mg./C c.	Nitrogen in Protective Dose	Dilutions of Samples 1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Precipitinogen	Precipitins (Samples as Antibody)
N/100 Na_2CO_3 (55 C. for 30 min.)	0.806	0.0025	S S	S S	S S	S S	S S	S S	S S	36 S	1:8	1:32
10% saccharose (55 C. for 30 min.)	0.147	0.015	S S	S S	44 60	36 44	44 S	36 36	36 36	36 36	0	0
10% saccharose and 9% NaCl (55 C. for 30 min.)	0.707	0.0022	24 36	S S	S S	S S	S S	S S	S S	48 S	1:16	1:32
		1:10,000,000								1:1,000,000		
Organism control....		24 36								36 36		

S indicates survival; numbers refer to hours of survival.

THE SOLUBILITY OF THE SPECIFIC PRECIPITATE AT VARIOUS HYDROGEN ION CONCENTRATIONS

Exper. 9.—The object of this experiment was to determine the hydrogen ion concentration of minimum solubility of the specific precipitate and to test the solutions of various hydrogen ion concentrations for protective value and agglutination as an index to the degree of dissociation. The material was prepared from 1 liter of a potent type 2 serum by the addition of sufficient type 2 soluble specific substance to make a concentration of 1 : 5,000. The precipitate was washed once with 0.85% sodium chloride solution and once with distilled water. It will be seen thus that the material contained some inorganic salts, although of low concentrations. The precipitate was taken up in 180 c c. of distilled water and dissolved with 40 c c. N/20 hydrochloric acid.

After standing a short time, the solution was centrifugalized to remove any insoluble material. It was then diluted with an equal volume of water, and 12 samples of 20 c c. each were withdrawn. For the estimation of the increment of sodium hydroxide to be added in the series, to one sample was added sufficient N/40 sodium hydroxide to cause precipitation and re-solution, 6 c c. being required. As it was thought 10 samples would give sufficiently close gradations in the series, the increment was found by dividing the total amount of alkali required by 10, giving 0.6 c c. However, in order to insure a closer approximation of the critical hydrogen ion concentration, during the experiment additional members of the series were added, namely, 2.1 c c. and 2.7 c c. After addition of the respective amounts of alkali, the samples were made up to equal volumes with water. To establish a solution equilibrium, the tubes were shaken 6 hours in a machine, after which they were centrifugalized, and the supernatants saved for tests.

The results, given in table 9, show that the hydrogen ion concentration of minimum solubility of the specific precipitate as indicated by nitrogen content, protective value, precipitin, and agglutination tests is at P_H 6.6. Irregularities occur in nitrogen content of the protective dose of different members of the series. One possible explanation of the variations is the wide difference in the dilutions used in estimating protective values. An error of this kind might possibly cause an apparent decrease, not over 50%, in the nitrogen content of the protective dose. It would thus be seen that the protein contained in a protective dose might well be more nearly constant than shown in the table. There is still another reason, which is perhaps a more plausible explanation; that is, both on the acid and on the alkaline side, there are two dissociated components, one the protective antibody, and the other the soluble specific substance which displays agressin-like or toxic action. As seen from table 9, this substance is present in greatest amount at P_H 4.4, and gradually decreases to the point of minimum solubility of the protein. Similar results are shown on the alkaline side at a hydrogen ion concentration of P_H 6.8.

It is possible that since the soluble specific substance has agressin-like action, the degree of dissociation can be measured by its effect in protection tests. For, as shown in table 9, there is present in a dilution of 1 : 5 of many of the samples sufficient soluble specific substance to inhibit the protective antibody. To measure the degree of dissociation, mice received injections with a constant dose of antibody (0.2 c c. of the undiluted sample) against variable doses of organism. The results of

this titration are definite; for it is seen in table 10 that the degree of dissociation measured in this manner corresponds to the precipitin tests shown in the previous table. The greatest amount of dissociation was in the P_H 4.4 sample, with a more or less gradual decrease in the middle range from P_H 6.4 to P_H 6.8. In this range of hydrogen ion concen-

TABLE 9

THE SOLUBILITY OF THE SPECIFIC PRECIPITATE AT VARIOUS HYDROGEN ION CONCENTRATIONS

All mice received injections of 0.5 c c. of an 8-hour broth culture diluted 1:200

Hydrogen ion Concentration	Volume NaOH, C c.	Nitrogen, Mg./C c.	Nitrogen in Protective Dose	Dilution of Supernatants 1:5	1:10	1:20	1:40	1:80	1:160	Precipitinogen	Agglutination (Supernatant as Antibody)
4.4	0.6	1.092	0.0680	24 36	S S	S S	S S	S S	S S	1:80	1:40
4.5	1.2	1.078	0.0672	24 S	S S	S S	S S	S S	S S	1:40	1:20
4.8	1.8	1.071	0.0668	S S	S S	S S	S S	S S	S S	1:40	1:20
5.1	2.1	0.595	0.0370	60 108	S S	S S	S S	S S	S S	1:20	1:10
5.3	2.4	0.300	0.0190	84 S	S S	S S	S S	S S	S S	1:10	1:5
6.4	2.7	0.073	0.0260	S S	S S	S S	24 36	20 36	18 22	1:2	0
6.6	3.0	0.022	.....	36 60	36 36	24 36	18 24	..	..	0	0
6.8	3.6	0.129	0.0260	S S	36 60	22 36	24 36	..	..	1:2	0
8.4	4.2	0.311	0.0390	60 S	S S	S S	S S	S S	60 96	1:10	1:2
9.2	4.8	0.616	0.0770	S S	60 120	S S	S S	S S	60 S	1:20	1:5
9.8	5.4	1.183	0.0739	22 22	22 36	84 84	S S	S S	S S	1:40	1:10
					1:10,000,000					1:1,000,000	
Organism control.....					24 24					36 36	

S indicates survival; numbers refer to hours of survival.

tration, failure to protect was due to an insufficient amount of antibody and not to the presence of the soluble specific substance. On the alkaline side, P_H 6.8 to P_H 8.4, the results are the same as on the acid side, but with the hydrogen ion concentration of P_H 9.2 and P_H 9.8 there is an apparent detoxification in a manner similar to the effect of sodium carbonate and heat shown previously.

The hydrogen ion concentration of minimum solubility of the protein combined with the soluble specific substance as given in this study lies in the same zone as that of the protective substance, separated from types 1, 2, and 3 immune serums by simple dilution with water.[24] Furthermore, at the hydrogen ion concentration of minimum solubility, both in the case of the specific precipitate and of the water-insoluble substance of types 1, 2, and 3 serums, the amount of protein present, though varying slightly, is of the same order, namely, containing from 0.02 to 0.04 mg. of nitrogen per c c. From this and other evidence, it is perhaps fair to make the assumption that the proteins separated from antipneumococcus serums by the soluble specific substances and those precipitated from these serums by simple dilution with water are similar, differing perhaps in the amount of soluble specific substance combined with the protective antibody.

PROZONE REACTION WITH SOLUBLE SPECIFIC SUBSTANCE

Exper. 10.—This experiment was planned to demonstrate the prozone reaction as determined by the amount of nitrogen and the protective value in the precipitate obtained with soluble specific substance. The antibody solution used was prepared from immune serum by dilution with water and purified by reprecipitations from sodium chloride solutions by dilution with water, the process being carried out 5 times. The following amounts of soluble specific substance, respectively, 100 mg., 10 mg., and 1 mg., were each dissolved in 10 c c. of 0.85% sodium chloride and then added to 10 c c. samples of the antibody solution. After thorough mixing, the tubes were incubated for 2 hours at 37.5 C., allowed to stand in the icebox over night, and centrifugalized. The supernatants only were used for tests. The amount of nitrogen in the precipitates was calculated by subtracting that found in the supernatants from that in the control. It is seen from table 11 that there was a pronounced zone effect. Precipitation occurred to a slight extent with 100 mg., more with 1 mg., and to the greatest extent with 10 mg. of the soluble specific substance. Protection tests parallel the amount of nitrogen in the precipitates. It is to be noted in this case also that, although all the protective substance was precipitated with 10 mg. of the specific substance, there remained in the supernatant 46% of the original nitrogen. Similar results concerning prozone reaction have been reported by Morgan,[2] who demonstrated that for optimum pre-

[24] Felton, L. D.: Bull. Johns Hopkins Hosp. In press.

TABLE 10

AN ANTAGONISTIC SUBSTANCE OF SPECIFIC PRECIPITATE AT VARIOUS HYDROGEN ION CONCENTRATIONS

All mice received injections of 0.2 c c. of undiluted samples

Hydrogen Ion Concentration	Dilution of Culture				
	1:10	1:100	1:1,000	1:10,000	1:100,000
4.4	20 60	24 36	84 108	S 84	36 108
4.5	22 S	44 68	S S	84 36	36 S
4.8	24 S	S S	S S	84 S	36 S
5.1	18 108	36 S	S S	S S	S S
5.3	108 84	S S	S S	S S	S S
6.4	18 23	S S	108 S	S S	S S
6.6	18 23	36 48	60 S	S S	S S
6.8	18 44	36 36	S S	S S	S S
8.4	18 36	108 S	68 S	S S	S S
9.2	60 104	S S	S S	S S	S S
9.8	70 S	S S	S S	S S	S S
	1:10,000,000			1:1,000,000	
Organism control	22 36			23 34	

S indicates survival; numbers refer to hours of survival.

TABLE 11

PROZONE REACTION WITH SOLUBLE SPECIFIC SUBSTANCE

All mice received injections of 0.5 c c. of an 8-hour broth culture diluted 1:100

Total Amount Soluble Specific Substance	Total Nitrogen of Supernatants	Total Nitrogen of Precipitates	Dilutions of Supernatants								
			1:2	1:5	1:10	1:20	1:40	1:60	1:80	1:100	1:200
100 mg.	19.10	0.34	S S	S S	S S	S S	36 40	20 60	20 S	44 60	36 36
10 mg.	8.96	10.48	72 36	18 80	36 36	18 18	36 36	36 36	20 36	18 36	20 21
1 mg.	10.58	8.86	84 S	S S	S S	96 84	36 44	S 36	36 36	36 36	36 36
Control	19.44	0.00	S S	S S	S S	S S	60 S	S S	S S	S S	36 S
				1:10,000,000				1:1,000,000			
Organism control.....				36 36					36 36		

S indicates survival; numbers refer to hours of survival.

cipitation from any amount of antipneumococcus serum there exists an optimum concentration of the soluble specific substance.

COMBINING WEIGHTS OF ANTIBODY AND SOLUBLE SPECIFIC SUBSTANCE

Exper. 11.—The previous experiment suggested a preliminary estimation of the combining weights of the antibody and specific sub-

TABLE 12

COMBINING WEIGHTS OF ANTIBODY AND SOLUBLE SPECIFIC SUBSTANCE

All mice received injections of 0.5 c c. of an 8-hour broth culture diluted 1:100

Amount Soluble Specific Substance, Mg.	Nitrogen of Supernatants, Mg./C c.	Nitrogen of Precipitates, Mg./C c.	Dilutions of Supernatants					Precipitinogen
			1:2	1:5	1:10	1:20	1:40	
10	9.52	5.16	20 S	44 60	36 40	36 36	24 36	1:250
5	8.62	6.06	18 S	36 S	18 36	36 36	24 36	1:24
3.3	8.68	6.00	48 S	S S	36 48	36 36	36 36	1:32
2.5	8.74	5.94	36 S	48 60	36 144	36 36	21 24	1:16
2.0	8.80	5.84	S S	68 S	S S	S S	S S	1:8
1.6	9.02	5.66	S S	S S	S S	S S	S S	
1.4	9.02	5.66	S S	S S	S S	S S	S S	1:4
1.2	9.08	5.60	S S	S S	S S	S S	S S	1:2
1.1	9.80	4.88	S S	S S	S S	S S	S S	1:2
1.0	10.24	4.44	S S	S S	S S	S S	S S	1:2
			1:20	1:40	1:80	1:160	1:320	
Antibody control	14.68	0.00	S S	S S	S S	S S	..	0
				1:10,000,000			1:1,000,000	
Organism control				36 36			36 36	

S indicates survival; numbers refer to hours of survival.

stance. The material used was from the same lot of antibody solution as in the preceding experiment. Other details were identical, with the exception of smaller differences in the amounts of soluble specific substance to insure a better end-point. The results, given in table 12, indicate that all of the protective material of this sample was precipitated

by 2.5 mg. of the specific substance. The end-point was judged from the lack of protective power of the supernatant together with a relatively small amount of free soluble specific substance as shown in the last column of the table. At this point it will be seen that 2.5 mg. of the specific substance precipitated protein containing 5.94 mg. of nitrogen. In another publication [24] it is reported that the precipitate obtained from immune serum by dilution with water may be freed from phosphorus by reprecipitation and yet be highly protective. This fact would indicate that we are at least dealing with a globulin different from euglobulin; for Chick [25] and others, more recently Sorensen,[26] reported essential phosphorus in the molecule of euglobulin. Hence, assuming that the protein concerned is perhaps of pseudoglobulin origin, and thus would have the molecular weight in the order of 80,000 reported by Sorensen [27] and confirmed by Cohn,[27] the combining ratio would be found by the following calculation:

$$2.5 : (5.94 \times 6.33) :: \times : 80{,}000$$
$$\times = 5{,}300 \pm$$

This calculation indicates that the molecular weight of the specific precipitate is in the order of 85,300 and the soluble specific substance is 5,300. These results are not given as a final determination, as our knowledge is lacking as to the chemical properties of the antibody. It is to be emphasized, however, that the specific precipitate is a combination, if not chemical in nature, of definite characteristics.

COMMENT AND SUMMARY

The results reported in this paper, as far as they are concerned with precipitation of the protective substance in antipneumococcus serums by bacterial products and subsequent dissociation, are a confirmation of the work of Gay and Chickering, Chickering, and Huntoon. The difference is the use of a single bacterial product for precipitation and control of the degree of dissociation by an estimation of the amount of protein in solution. We have extended the work of these investigators in a more complete study of the characteristics of the precipitate, and, further, made attempts to separate the products of dissociation; i. e., the precipitating agent from the antibody.

[25] Biochem. Jour., 1914, 8, p. 404.
[26] Jour. Am. Chem. Soc., 1925, 47, p. 457.
[27] Jour. Biol. Chem., 1925, 63, p. 721.

Our results may be summarized briefly as follows: With appropriate amounts of the soluble specific substance, all the protective substance in type 2 antipneumococcus serums may be precipitated. The precipitate so obtained is practically insoluble in water, sparingly soluble in 3% sodium chloride, and very soluble in dilute acid and alkali when the salt content is low. The hydrogen ion concentration of minimum solubility of the one sample tested is P_H 6.6. Assuming that the protein of the protective substance has the same molecular weight as pseudoglobulin, the combining ratio per weight of soluble specific substance to protein is 1 : 15, the molecular weight of the soluble specific substance being of the order of 5,300 and the total molecular weight of the resulting combination 85,300.

Sodium carbonate in concentration of M/100 is the best of the dissociating agents used (also Chickering, Huntoon, Berger). Dissociation takes place readily in dilute acid and alkali solutions (hydrochloric acid and sodium hydroxide) as judged by free soluble specific substance, but the resulting solutions have no demonstrable protective value. However, if to the acid solution is added sodium chloride to make 25% saturation, a precipitate is thrown down containing the protective substance. Also when a sample of precipitate is dissociated by heat in the presence of sodium carbonate, disodium phosphate, saccharose, or sodium chloride, and to the clear solution sodium sulphate is added to make 18% practically all the protective substance is precipitated. However, this fraction insoluble in 18% sodium sulphate still contains a small amount of material antagonistic to defense, as well as a highly protective substance.

INFLUENCE OF SOME ANAEROBIC SPECIES ON TOXIN OF CL. BOTULINUM WITH SPECIAL REFERENCE TO CL. SPOROGENES *

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The effect which Cl. sporogenes has on Cl. botulinum when grown in mixed culture has been pointed out in a previous paper.[1] The object of this investigation was to study the effect of Cl. sporogenes on the toxin of Cl. botulinum and to determine the effect of sporogenes filtrates on Cl. botulinum.

Attention has been called to Cl. sporogenes as a contaminant in cultures of Cl. botulinum by numerous workers in anaerobic bacteriology. Reddish,[2] a few years ago, found 18 out of 19 strains of Cl. botulinum obtained in this country to be contaminated with Cl. sporogenes. Such contaminations might well account for many of the strains of Cl. botulinum which were supposed to have lost their toxin-producing properties.

The effect of some aerobic species of organisms on the toxin of Cl. botulinum was studied by Hall and Peterson.[3] They found that impure aerobic cultures from certain soils would inhibit the toxin production of Cl. botulinum in glucose broth and that certain acid-producing aerobes would inhibit toxin production in glucose broth but not in plain broth. The acidity alone could not be used in accounting for the toxin destruction.

The ability of anaerobes to destroy toxins has not been studied extensively. Many investigators have noticed that Cl. botulinum cultures heavily contaminated with Cl. sporogenes are slightly toxic and often nontoxic. Donaldson,[4] in working with the "Reading Bacillus," which he found closely related to Cl. sporogenes, observed that it would destroy tetanus and diphtheria toxin in large amounts. He used this organism in treating war wounds. In his conclusions, he makes the following statements:

> The rationale of the method depends not on the inhibition by the Reading bacillus of the growth of pathogenic organisms in the wound, either by reason

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[1] Jordan, E. O., and Dack, G. M.: Jour. Infect. Dis., 1924, 35, p. 576.
[2] Jour. Infect. Dis., 1921, 29, p. 120.
[3] Jour. Bacteriol., 1923, 8, p. 319.
[4] Jour. Path., 1918, 22, p. 129.

of the formation by the bacillus of any inhibitory organic acid, or by the production of any bacteriolytic ferment, as symbiotic experiments show. . . . Not only so, but it is probably able to hydrolyze the toxic degradation products of other organisms. In support of this a résumé is given of experiments on tetanus and other toxins, which show that the Reading bacillus, out of a series of organisms investigated, is alone able to reduce the toxicity of these toxins. There is one exception, namely, B. sporogenes (Metchnikoff), which, however, does not appear to be so potent in this direction as the Reading bacillus. . . .

TABLE 1

DESTRUCTION OF CL. BOTULINUM TOXIN BY CL. SPOROGENES

Dilution	Toxin after Incubation of																			
	24 Hours				48 Hours				72 Hours				5 Days				11 Days			
	1	2	C1	C2	1	2	C1	C2	1	2	C1	C2	1	2	C1	C2	1	2	C1	C2
1:50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:100	+	+	+	+	+	+	+	+	+	+	+	+	+	S*	+	+	+	0	+	+
1:500	+	+	+	+	..	..	+	+	0	0	+	+	0	0	+	+	0	0	+	+
1:1,000	+	+	+	+	+	0	+	+	0	0	+	+	0	0	+	+	0	0	+	+
1:5,000	+	0	+	+	0	0	+	+	0	0	+	+	0	0	0	+	0	0	0	0
1:10,000	0	0	+	+	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0

* S indicates symptoms.
C indicates control toxin tubes; P_H of control toxin tubes, 6.9; P_H of control tube on uninoculated medium, 7.2; P_H of sporogenes-toxin mixtures, 7.5.

TABLE 2

DESTRUCTION OF CL. BOTULINUM TOXIN BY CL. SPOROGENES

Dilution	Toxin after Incubation of															
	48 Hours				80 Hours				5 Days				10 Days			
	1	2	C1	C2	1	2	C1	C2	1	2	C1	C2	1	2	C1	C2
1:10	+	+	..	..	+	+	..	..	+	+	..	..	+	+	..	..
1:50	+	+	..	..	+	+	..	..	+	+	..	..	0	+	..	..
1:100	+	+	+	+	0	+	+	+	0	+	+	+	0	+	+	+
1:500	..	..	..	..	..	..	..	..	..	..	..	..	0	0	..	..
1:1,000	0	0	+	+	0	0	+	+	..	..	+	+	..	..	+	+
P_H	7.4	7.5	7.3	7.4	7.4	7.5	7.3	7.4	7.4	7.5	7.2	7.3	7.0	7.1	6.9	7.0

C indicates control toxin tubes.

EFFECT OF THE GROWTH OF CL. SPOROGENES ON BOTULINUM TOXIN IN BEEF HEART MEDIUM

The beef heart medium was prepared in the usual way with few modifications. The meat was first dried in the oven and 0.7 gm. amounts were placed in tubes and covered with 10 c c. of broth. The medium was sterilized by autoclaving at 18 pounds' pressure for 1 hour, after which it was incubated at 37 C. for several days to insure sterility. One c c. portions of sterile botulinum toxin, type A ($M7a^2$) were added to the medium after it had been reheated and cooled. The tubes were then inoculated with Cl. sporogenes and sealed with sterile petrolatum.

The toxin was titrated at intervals, as shown in the tables. The hydrogen ion concentrations in one experiment were determined at the same time that

the titration of the toxin was made. In the other experiments, it was determined at the end of the period (tables 1 and 2).

In table 1, when a toxin of high potency was used, it will be noted that the potency of the toxin in the control tubes had decreased 10 times that of the titer at the start of the experiment. This toxin was freshly prepared and was not as stable as the older toxin used in the experiment shown in table 2.

Experiments were made to determine the effect of temperature on the titer of toxin in tubes identical to the controls shown in tables 1 and 2. An old toxin and one freshly prepared were used. The incubation was carried on at refrigerator temperature, 6 to 7 degrees in one case and at 37 C. in the other.

From previous work it was found that an old toxin (stored 2 months in the refrigerator) which is more or less stabilized does not lose considerably in potency over the 10 day incubation period at 37 C.; in no

TABLE 3

EFFECT OF TEMPERATURE ON BOTULINUM TOXIN IN BEEF HEART MEDIUM

Tube No.	Incubation Temperature	M L D						
		Before Incubation			After 10 Days Incubation			
		1:1,000	1:2,500	1:5,000	1:100	1:250	1:1,000	1:2,500
1	37 C.	+	S	S	+	0	0	0
2	6-7 C.	+	+	S	+	+	+	0

The toxin used in the above tubes was freshly prepared and of a high titer.
S indicates symptoms.

case was there a reduction greater than 2 times the original titer of the toxin mixture. In the freshly prepared toxins as much as 10 times the original titer of the toxin is lost over the same period of time (table 3). Little loss in potency was shown in either the new or the old toxin mixtures when the tubes were kept at refrigerator temperature.

As may be seen from the tables, a marked reduction in the potency of the toxin occurred during the growth of Cl. sporogenes. The drop in titer was evident after 24 hours, and the reduction continued over the whole period of the experiment. The destruction of toxin by the strain of Cl. sporogenes used was a slow process, but seemed to be a progressive and gradual one.

EFFECT OF OTHER ANAEROBIC SPECIES ON BOTULINUM TOXIN IN BEEF HEART MEDIUM

The medium used in these experiments was prepared in the same manner as that used in the previous experiments. A 16-hour incubation period was allowed to elapse after adding the toxin as an extra precaution to insure sterility

and allow the toxin to diffuse properly through the medium. Mice received injections of 1 c c. amounts of 1 : 10 and 1 : 100 dilutions from each of the tubes. The 1 : 100 dilution was found to be sufficient to kill mice in every case.

The cultures used in the experiments were obtained from different laboratories in this country. They were first purified by single cell isolation and later tested in various proteolytic mediums to check their cultural characteristics. With but two exceptions, the organisms checked fairly closely with the characteristics ascribed to them. The two exceptions were R 20 c which was an isolation from a culture labeled "B. welchii"—it was proteolytic and nonsaccharolytic; and R 21 c, which was an isolation from a culture labeled "Cl. sporogenes," which proved to be only slightly proteolytic and was a nonfermenter.

TABLE 4

FALL IN TITER OF BOTULINUM TOXIN IN BEEF HEART MEDIUM INOCULATED WITH ANAEROBES

Cultures	Toxin								
	10 Days					41 Days			
	Undiluted	1:10	1:100	1:500	1:1,000	Undiluted	1:10	1:100	P_H
R34a Cl. bifermentens....	+	+	0	..	..	0	0	..	7.4
R33 Cl. fallax.....	+	+	±	..	..	0	..	..	7.2
R12c Cl. flabelliforme.....	+	+	0	..	..	0	0	..	7.6
R7b Cl. histolyticum.....	+	+	0	..	..	0	0	..	7.2
R31c Cl. parasporogenes.	+	0	0	..	..	0	0	..	6.9
*R11b Reading bacillus....	+	+	0	..	..	0	0	..	7.3
R1b Vibrion septique....	+	+	+	..	..	+†	+	0	7.2
*R6a Cl. sporogenes.....	+	+	0	.	..	0	0	..	7.2
*R6b Cl. sporogenes.....	+	+	0	..	..	0	0	..	7.2
R18d Cl. sporogenes.....	+	+	0	..	..	0	0	..	7.6
R21c Cl. sporogenes.....	+	+	0	..	..	0	0	..	7.0
R22a Cl. sporogenes.....	+	+	0	..	..	0	0	..	6.6
*R23e Cl. sporogenes.	+	+	0	..	..	0	0	..	7.2
R26c Cl. sporogenes.....	+	+	0	..	..	0	0	..	7.0
R27d Cl. sporogenes.....	+	+	0	..	..	0	0	..	7.3
*R8d Cl. tetanomorphus..	+	+	0	..	..	0	0	..	7.1
*R20c Cl. welchii.....	+	+	0	..	..	0	0	..	7.3
R4b Cl. welchii.....	+	+	+	..	..	+†	+	0	7.0
Control.....	+	+	+	+	0	+	+	+	7.0
Control.....	+	+	+	+	0	+	+	+	7.0

* Duplicates were made.
± Died on the seventh day.
† Antitoxin controls receiving 0.25 c c. of undiluted culture plus 0.25 c c. type A botulinus antitoxin; did not die.

The inoculations were made from beef heart cultures. The tubes were incubated for 10 days at 37 C. before the first test was made. Before placing them back in the incubator after making the first test, they were left at room temperature for 2 days.

The centrifuged supernatant fluid was injected into mice. The mice receiving the undiluted fluid were given 0.25 c c., and those receiving the dilutions were injected with 1 c c. portions. Tests were run after 10 and 41 days (table 4).

Several sporogenes strains were selected in order to check the reactions obtained with the 310-1 strain and to determine whether or not there was any variation among them as regards their ability to destroy toxin.

Most of the strains employed were proteolytic, some of them more so than others. There were three notable exceptions which were nonproteolytic, namely, Cl. fallax, Vibrion septique and Cl. welchii. In the 10 day interval the tubes inoculated with the nonproteolytic organisms were the only ones which proved to be toxic in the 1 : 100 dilution. After the 41-day period, there was no demonstrable toxin in most of the tubes. The controls contained just enough toxin so that the animals receiving the 1 : 100 dilutions died within the 2 day period. Toxin in the Cl. fallax culture had completely disappeared.

The Cl. welchii (R4b) and Vibrion septique (R1b) cultures were toxic in the 1 : 10 dilution after 41 days of incubation. Since many of the cultures used were nonpathogenic, no antitoxin controls were run at the 10 day interval except in the case of the uninoculated control tubes, so that it was not known whether the toxic action of the Vibrion septique and Cl. welchii cultures was due to the toxins of the respective organisms inoculated or to the botulinum toxin. The toxin destruction was more marked in those cultures after the 41-day period, as shown in the table.

The destruction of toxin occurred in every tube into which organisms were inoculated. The toxin destruction was more marked in those cultures which possessed some proteolytic activity. In cultures of Vibrion septique, Cl. welchii, and Cl. fallax, the destruction was much slower, and in the case of the two former organisms was not complete after 41 days of incubation.

The ability of these organisms to destroy toxin cannot be taken as an index of their behavior when actively associated with Cl. botulinum. In the isolation of Cl. botulinum from soils or various other sources by using the beef heart enrichment method, one could readily understand from these experiments, and from the ones previously reported [1] the reasons for obtaining very weak toxins which may disappear before the sample can be typed or the injections repeated a few days later.

GROWTH AND TOXIN PRODUCTION OF CL. BOTULINUM IN A SPOROGENES FILTRATE

The sporogenes filtrate was obtained from a beef heart culture of Cl. sporogenes (310-1) which had been incubated for 12 days at 37 C. The supernatant fluid was removed from the culture and filtered through a Berkefeld filter. The filtrate was tested both aerobically and anaerobically for sterility.

The spore suspensions used in the experiments were prepared for both "A" and "B" types of Cl. botulinum. They were prepared as follows: Inoculations were made into casein digest medium (alkaline egg) of M7a[2] (type A) and 126.7 (type B) organisms of Cl. botulinum. These organisms were from

single cell strains. After a suitable incubation period, growth in this medium had ceased, and the organisms settled out, leaving a clear supernatant fluid which was pipetted off. The sediment containing the spores was washed 3 times with sterile 0.85% sodium chloride solution. During centrifugalization the tube containing the M7a² (type A) suspension was broken. M7a² organisms taken from an old casein digest broth culture were substituted. The culture was 7 months old and stood in the incubator for some time. The organisms taken from this culture were diluted with 0.85% sodium chloride solution and used immediately.

Both suspensions were heated to 75 C. for 10 minutes. One half c c. portions were injected intraperitoneally into mice to test for the presence of toxin. The mice were kept on test for a period of a week, and during this time showed no symptoms. The suspensions were used immediately after testing in mice.

TABLE 5

Growth of Cl. Botulinum in Sporogenes Filtrate

Sporogenes Filtrate 310-1	0.1 C c. Toxin-Free Spore Suspension of Cl. Botulinum	Treatment	Examination after 10 Days' Incubation at 34 C.			
			Count per C c.	Toxin	Growth of Organisms	P_H
3 c c.	M7a²	.....	24,000	±*	+	7.4
3 c c.	M7a²	.....	16,000	±†	+	7.4
3 c c.	126.7	.....	53,000	0	0	7.4
3 c c.	126.7	.....	52,000	0	0	7.4
3 c.c.	.....	.....	.....	..	..	7.5
3 c c.	M7a²	Heated 56 C., 30 min.	9,000	+	+	7.4
3 c c.	M7a²	Heated 56 C., 30 min.	15,000	+	+	7.4
3 c c.	.....	Heated 56 C., 30 min.	.....	..	..	7.5
3 c c.	M7a²	Heated 70 C., 30 min.	28,000	+	+	7.4
3 c c.	M7a²	Heated 70 C., 30 min.	19,000	+	+	7.4
3 c c.	.....	Heated 70 C., 30 min.	.....	..	..	7.5
3 c c.	M7a²	Boiled 10 min.	108,000	+	+	7.4
3 c c.	M7a²	Boiled 10 min.	170,000	+	+	7.4
3 c c.	.....	Boiled 10 min.	.....	..	..	7.5
	M7a²	3 c c. beef heart broth	∞	+	+	7.8
	126.7	3 c c. beef heart broth	∞	+	+	7.6

M7a² indicates type A botulinum—0.1 c c. contained 3,000 organisms; 126.7, type B botulinum —0.1 c c. contained 200,000 organisms.
* Died in 48 days.
† Died in 4 days.
∞ Profuse growth.

Counts were made of the suspensions in deep agar shakes, after 2 to 3 days' incubation at 35 C. The M7a² suspension contained 30,000 organisms per c c. and the 126.7 suspension 2,000,000 organisms per c c. Only 0.1 c c. portions of these suspensions were used in the inoculums.

Three c c. portions of the sporogenes filtrate were pipetted accurately into each of 14 tubes. Some of the tubes containing the filtrate were heated at various temperatures before adding the suspensions. Three c c. amounts of sterile beef heart broth were pipetted into 2 tubes which were to be used as controls. For the protocol of the experiment see table 5.

The tubes after inoculation with the suspensions were placed in a desiccator which was immediately evacuated, the pressure being reduced to 1.2 cm. of mercury. No leaks were found at the end of the 10-day incubation period.

A very heavy clouding occurred in the two control tubes within 48 hours' incubation. Much settling out of the organisms in these tubes was noted at the close of the incubation period.

No visible signs of growth could be detected in any of the tubes containing the filtrates. Quantitative dilutions were made in water blanks, and 1 c.c. portions of these dilutions were plated according to Krumweide's method, and counts made after 48 hours of incubation. Although the methods of counting viable anaerobes are not accurate, they serve a useful purpose in showing whether or not growth has occurred in the tubes even though it is not visible macroscopically.

The hydrogen ion concentration fluctuated little, and was suitable for the growth of Cl. botulinum. In the case of Cl. botulinum type B, no growth was noted, and associated with this no toxin was demonstrated.

In the heated filtrates which were boiled for 10 minutes, the organisms had increased in numbers. This is in agreement with Felton's [5] findings in regard to the growth of certain aerobes on reinoculated mediums. He makes the following statement in his conclusions: "Sterilization by heat seemed to render the food supply of metabolized media more available for bacterial consumption."

In the filtrates heated to 56 C. and to 70 C. for 30 minutes, there seemed to be no apparent increase over that of the unheated filtrates.

It is interesting to note the increased strength of the toxin in the heated tubes over that in the unheated tubes. Animals receiving the heated filtrates to which Cl. botulinum ($M7a^2$) type A were added, all died in less than 24 hours, whereas from 1 to 3 days longer were required when no heating was given. This apparent difference in toxicity does not seem to be associated with the multiplication of the organisms.

Antitoxin controls were run on all of the filtrates, and none of the protected mice died. No quantitative titrations of the strength of the toxins were made, hence no comparisons can be made of the potency of the toxins in the various tubes. However, it is obvious that heating the sporogenes filtrate favors the development in it of a stronger toxin by the Cl. botulinum.

A marked inhibition of growth occurred throughout the series in which the sporogenes filtrate was used. A study of the chemical nature of the sporogenes filtrate is necessary in order to elucidate its growth inhibitory properties for Cl. botulinum. At the present time no such study has been made.

Sporogenes organisms inoculated into sterile botulinum toxin were found not to destroy it in 7 days' incubation at 37 C. No growth was

[5] Bull. Johns Hopkins Hosp., 1923, 34, p. 313.

visible in the tube containing the mixture. Tests were not made to determine whether any multiplication had occurred or whether the toxin had fallen in titer.

The fact that there was an inhibition of growth parallels the findings in the case of the sporogenes filtrate which was inoculated with Cl. botulinum. However, as shown in tables 2 and 3, when the toxin is diluted about 1 : 10 in fresh medium, the Cl. sporogenes apparently is not inhibited in its growth.

EFFECT OF SPOROGENES FILTRATE ON BOTULINUM TOXIN

The filtrates of Cl. sporogenes and Cl. botulinum cultures used in this experiment were prepared from the same batch of beef heart medium. The incubation time of the inoculated medium was 12 days at 35 C.

One c c. portions of the filtrates were tested aerobically and anaerobically for sterility. After mixing the filtrates in the proportions as recorded in table 6, a preliminary titration of the toxin was made.

TABLE 6

M L D of Filtrate Mixtures Before and After Incubation

Filtrate	Before Incubation			After Incubation					
	1:1,000	1:10,000	1:100,000	1:100	1:500	1:750	1:1,000	1:1,250	P_H
2 c c. 310-1, 2 c c. M7a²	+	—	—	+	±	—	—	—	7.5
2 c c. 310-1, 2 c c. M7a²	+	+	—	+	±	±	—	—	7.5
M7a² control	+	+	—	+	+	..	S	—	7.5

±, died in 3 to 4 days. S, symptoms in 48 hours, lived.

After withdrawing sufficient samples for the titration, the tubes were placed in the desiccator along with the tubes containing the sporogenes filtrates as described in the preceding experiments. The incubation was carried out at 35 C. for 10 days, following which the tubes were removed and the contents titrated. Hydrogen ion determinations were also made. The results are recorded in table 6.

From table 6 it may be seen that the sporogenes filtrate had little if any effect on the potency of the botulinum toxin even after 10 days' incubation. The loss in potency after the incubation is comparable to that of the freshly prepared toxin, as shown in table 3.

SUMMARY

The filtered toxin of Cl. botulinum added to beef heart medium is gradually destroyed by the growth of Cl. sporogenes in it.

The power to destroy botulinum toxin is also possessed by several other anaerobic species with proteolytic and nonproteolytic properties.

The destruction of the toxin is not due to a change in hydrogen ion concentration.

Cl. botulinum type A ($M7a^2$) grew scantily in a filtrate of Cl. sporogenes. Growth was more vigorous in a filtrate which had been boiled but still was invisible macroscopically. Toxin was demonstrated in all the tubes but was stronger in the tubes containing the filtrate which had been heated.

Cl. botulinum type B (126.7) did not grow in the unheated sporogenes filtrate nor did it produce toxin.

No reduction in potency of the toxin of Cl. botulinum type A ($M7a^2$) was noted when an equal amount of sporogenes filtrate was added to it.

No visible growth (macroscopically) of Cl. sporogenes occurred in a filtrate of Cl. botulinum after 7 days' incubation at 37 C.

BEHAVIOR OF BOTULINUM TOXIN IN ALIMENTARY TRACT OF RATS AND GUINEA-PIGS *

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The behavior of botulinum toxin introduced into the alimentary tract is different from that in other portions of the body. Mice will tolerate large doses of the toxin given orally without showing any symptoms of botulism, but they are susceptible to the toxin injected intraperitoneally, even in high dilutions. Similar differences more or less pronounced are noted in rats and other animals.

The object of this investigation is to compare the behavior of the toxin in animals resistant to oral injections with that in animals that are susceptible. Guinea-pigs and rats were used for the most part, but in one series guinea-pigs and mice were compared.

The general literature has been well reviewed by Bengtson.[1] Reference will be made here only to the literature bearing on the particular subject of this paper.

Observations on the absorption of botulinum toxin by the tissues have been negative, except in the case of the nervous tissue. Kempner and Schepilewsky [2] found that emulsions of liver, kidney, spleen and muscle tissue from the guinea-pig did not absorb toxin, but that 1 c c. of brain emulsions would neutralize 3 to 4 minimal lethal doses of toxin if mixed in vitro. Schübel,[3] in working with tissue emulsions from the guinea-pig and rabbit, found the same thing. He used frogs in testing for toxin, and noted that emulsions of brain and toxin after a suitable incubation period required much longer to produce the characteristic symptoms than was the case with the other tissue emulsions and toxin.

The behavior of botulinum toxin in different portions of the intestine in vivo has been studied by several workers. Charrin and Claude [4] injected botulinum toxin into loops of intestines of guinea-pigs and rabbits and later made a histologic examination of the tissue. They found that the intestinal muscosa had remained intact. Their findings are

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[1] Hygienic Lab. Bull., 1924, No. 136.

[2] Ztschr. f. Hyg. u. Infectionskrankh., 1898, 27, p. 213.

[3] Arch. f. exper. Path. u. Pharm., 1923, 96, p. 193.

[4] Arch. de Pharmacodynamie, 1898, 4, p. 491.

briefly: (1) intact epithelium, (2) round cell infiltration into glands and submucosa, (3) dilatation of blood vessels.

Forssman[5] found that there was no absorption of the toxin when it was directly introduced into the cecum or large intestine. He also found that 10 gm. of cecal contents of a rabbit would "precipitate" 1,000 minimal lethal doses of toxin. When he introduced the toxin through a catheter or by laparotomy into the small intestines, he noted the same symptoms as he obtained by giving subcutaneous injections, but larger amounts were required.

Little work has been done to show the presence and persistence of toxin in the blood of animals receiving injections with botulinum toxin. Van Ermengem[6] introduced 5 c c. of toxin into the stomach of a guinea-pig and was able to demonstrate toxin in the circulating blood after 30 minutes but not after 15 minutes. He injected the blood into mice in 0.5 c c. amounts. Kob[7] was able to produce symptoms of botulism in mice by injecting into them the blood of a patient taken 9 days after the poisonous food was eaten. Bengtson[8] was able to demonstrate toxin in 0.1 c c. of citrated blood but not in 0.01 c c. from a guinea-pig which had fed on some cabbage that had been sprayed with 3 c c. of a 9 day glucose broth culture of Cl. botulinum. Apparently, no definite measurement was made of the amount of toxin the animal had consumed.

EFFECT OF COMMINUTED GASTRIC TISSUES AND THEIR EXTRACTS ON THE TOXIN OF CL. BOTULINUM IN VITRO

A female guinea-pig weighing about 600 gm. was killed by a blow on the head. The stomach was immediately removed and cut longitudinally into two equal portions. The contents were removed, and the pieces rinsed in tap water.

One half of the stomach was ground with sterile sand in a mortar and suspended in 5 c c. of water. It was then centrifugalized and 2 c c. of the supernatent fluid withdrawn and used in the tests. The other half was cut into small pieces (minced) and placed in a tube. To it was added 2 c c. of sterile distilled water.

A control observation was made, taking about 2 square inches of the muscle and peritoneum from the abdominal wall. This tissue was divided in half and treated in a manner identical to that described for the stomach.

Five normal mice were killed by a blow on the head. The stomachs were immediately removed and treated in a manner similar to that described for the stomach of the guinea-pig, with the exception that 5 stomachs were used in order to make the volume comparable to that employed above. About 1 square inch of muscle and peritoneum was removed from the abdominal wall of each

[5] Cited by Bengtson, Ref. 1.

[6] Arch. de Pharmacodynamie, 1897, 3, pp. 213, 499.

[7] Cited by Bengtson, Reference 1.

[8] Public Health Rep., 1921, 36, p. 1665.

mouse as a control. These tissues were treated as in the guinea-pig, the volumes of diluting fluid remaining the same.

One c c. of sterile botulinum toxin, type A ($M7a^2$), was added to each tube and also to a control tube containing 2 c c. of sterile distilled water. The tubes were placed in the incubator at 35 C. for one hour. They were then removed, and dilutions of 1 : 10, 1 : 50, 1 : 100, 1 : 500 and 1 : 1,000 were made. Control mice were inoculated with 0.25 c c. of the undiluted fluid plus 0.25 c c. of the homologous antitoxin. Hydrogen ion determinations were made of the mixtures. The results are shown in table 1.

As shown in the table, the potency of the toxin was altered only in the case of the stomachs of mice, in which it was reduced to about one half the original titer. It is doubtful whether this is of any significance in explaining the resistance of mice to oral injections of the toxin.

TABLE 1

The Effect of Comminuted Gastric Tissues and Their Extracts on the Toxin of Cl. Botulinum in Vitro

Animal	Tissue	Treatment of Tissue	P_H	Titer of Toxin After 1 Hour's Incubation at 35 C.
Guinea-pig	Stomach	Ground	7.4	1:1000
Guinea-pig	Stomach	Minced	7.3	1:1000
Guinea-pig	From abdominal wall	Ground	7.1	1:1000
Guinea-pig	From abdominal wall	Minced	6.7	1:1000
Mouse	Stomach	Ground	7.5	*1:100
Mouse	Stomach	Minced	7.4	1:500
Mouse	From abdominal wall	Ground	7.5	1:1000
Mouse	From abdominal wall	Minced	7.4	1:1000
Control toxin 2 c c. sterile distilled water plus 1 c c. botulinum toxin			7.8	1:1000

* The mouse receiving the 1:500 dilution of this mixture showed severe symptoms in 2 days, but did not die.

BOTULINUM TOXIN IN A LIGATED LOOP OF SMALL INTESTINE IN A GUINEA-PIG

A right rectus incision was made on a male guinea-pig weighing 975 gm. A ligature was placed around the intestine, between the blood vessels and intestine, about 18 inches above the cecum. Another ligature was similarly placed about 4 inches further up. One c c. of sterile botulinum toxin type A ($M7a^2$) was injected into this segment, a gage number 27 needle being used to make the injection.

The toxin employed was quite old, having been stored on ice for 59 days. The MLD for mice was 1 : 2,500. After injection the animal was kept on the operating board for 3 hours. No ether was given during this interval. At the end of this period, the animal was placed under ether and 0.2 c c. of fluid was withdrawn from the loop contents. Although the volume of the material in the enclosed segment was estimated at 2 c c., no more fluid could be withdrawn as the needle plugged with the solid contents in the loop. The ligatures were removed and the incision closed. The animal died during the night. Necropsy was made, but no significant findings were observed. There was no peritonitis or evidences of injury.

The fluid withdrawn after 3 hours was diluted with 1 c c. of sterile distilled water and centrifugalized at high speed for 2 hours. After centrifugalizing, the clear supernatent fluid was withdrawn, diluted, and 1 c.c. of the dilutions injected intraperitoneally into white mice. Dilutions of 1 : 5, 1 : 10, 1 : 50 and 1 : 100 were made. The mice receiving the 1 : 5 and 1 : 10 dilutions both died, the others failing to show symptoms. A control mouse was injected with 0.5 c c. of the 1 : 5 dilution plus 0.25 c c. of the homologous antitoxin. This mouse showed no symptoms.

BOTULINUM TOXIN IN THE INTESTINES OF GUINEA-PIGS AND RATS

The animals used in these experiments were placed on a milk diet 48 hours before the operation. A left rectus incision was made, and the small intestine was ligated about one-half inch from the cecum. One c c. of a weak toxin with an MLD of 1 : 1,000 for mice was injected into the stomachs with the use of a narrow gage needle. At intervals of 1, 3 and 12 hours, the animals were killed by a blow on the head, and the entire small intestines and stomach were removed. Two guinea-pigs and 2 rats were used for each of the intervals.

TABLE 2

PERSISTENCE OF BOTULINUM TOXIN IN THE STOMACH AND SMALL INTESTINES OF GUINEA-PIGS AND RATS RECEIVING INJECTIONS OF TOXIN DIRECTLY INTO THE STOMACH

Animal	Interval in Hours	Material Injected	Toxin			Antitoxin Control
			Undiluted	1:10	1:100	
Guinea-pig 1	1	G. I.	+	+	S	D.
Guinea-pig 1	1	W.	+	+	0	D.
Guinea-pig 2	1	G. I.	+	0	0	Lived
Guinea-pig 2	1	W.	+	0	0	Lived
Rat 1	1	G. I.	+	0	0	Lived
Rat 1	1	W.	+	0	0	Lived
Rat 2	1	G. I.	+	0	0	Lived
Rat 2	1	W.	+	+	0	Lived
Guinea-pig 3	3	G. I.	+	0	0	Lived
Guinea-pig 3	3	W.	0	0	0	Lived
Guinea-pig 4	3	G. I.	+	+	0	D.
Guinea-pig 4	3	W.	+	+	0	Lived
Rat 3	3	G. I.	+	0	0	D.
Rat 3	3	W.	+	+	0	Lived
Rat 4	3	G. I.	+	0	0	Lived
Rat 4	3	W.	+	+	+	D.
Guinea-pig 5	12	G. I.	0	0	0	Lived
Guinea-pig 5	12	W.	+	0	0	Lived
Guinea-pig 6	12	G. I.	0	0	0	Lived
Guinea-pig 6	12	W.	+	0	0	Lived
Rat 5	12	G. I.	0	0	0	Lived
Rat 5	12	W.	+	0	0	D.
Rat 6	12	G. I.	0	0	0	Lived
Rat 6	12	W.	+	0	0	D.

G. I. indicates ground intestine; W., washings from intestine; S., symptoms of botulism; D., death of antitoxin control mouse.

The fluid contents of the stomach and intestines were squeezed out and placed in centrifuge tubes. When little or no fluid was found in the stomach and intestines, 5 c c. of distilled water was used in rinsing them out. The washings were placed in centrifuge tubes. The stomach and small intestine remaining were ground up in a mortar with sterile sand and 5 c c. of sterile distilled water. After thorough grinding this material was placed in separate centrifuge tubes. The washings and ground stomachs and intestines were then centrifuged at high speed for an hour. The supernatent fluid was withdrawn, diluted and injected into mice. The results are shown in table 2.

An antitoxin control mouse which received 1 c c. of the undiluted centrifugalized supernatant fluid plus 0.25 c c. of the homologous antitoxin was used in each titration. As may be seen from table 2, several of the antitoxin control mice died, which indicated that the intestinal organisms present were not sufficiently thrown out of solution to prevent peritonitis; however in only one case (guinea-pig 1) did both of the antitoxin control mice die.

There was apparently some loss in potency of the toxin. The significant fact, however, is that the toxin remained in the intestinal tract of guinea-pigs for at least 12 hours. Both of the control mice receiving the intestinal washings from the rats in the 12 hour series died, so that it is not known whether or not they contained botulinum toxin. In these experiments, there was no way of knowing how much of the toxin was absorbed or how much of it might have undergone destruction in the intestine.

BOTULINUM TOXIN IN THE BLOOD OF GUINEA-PIGS AND RATS FED WITH THE TOXIN

Toxin produced in beef heart medium from a type A culture ($M7a^2$) was used in these experiments. The toxin fed to guinea-pig 1 and rat 1, as shown in tables 3 and 4, was old and of a low titer, 1 c c. of a 1 : 1,000 dilution given intraperitoneally being the MLD for mice. A toxin of greater potency was used in the remaining experiments, the MLD for mice of the latter toxin being 1 : 25,000. A small rubber urethral catheter was used in feeding guinea-pigs. Rats were fed with a silver Eustachian catheter. Quantities of toxin varying from 1 c c. to 10 c c. were fed.

The blood of normal rats and guinea-pigs was injected into mice intraperitoneally, without any harmful effects. Doses as large as 1 c c. were given. The blood was drawn by making cardiac punctures and was injected into mice immediately before it had time to clot. When more than 1 c c. of blood was drawn at one time, it was centrifuged and the serum injected (tables 3 and 4).

In one instance a larger quantity of blood was drawn and injected directly into guinea-pigs weighing about 200 gm. It was hoped that by so doing smaller amounts of toxin could be detected in the blood. Only 3 guinea-pigs received injections, 2 with the blood from guinea-pigs, as shown in table 3, and 1 with the blood from a rat, as shown in table 4.

Animals were bled at various intervals after feeding them the toxin and in every case when showing marked symptoms. In only 2 cases out of 7 was toxin demonstrated. Guinea-pigs 6 and 7 were fed at the same time and

TABLE 3

INJECTION INTO MICE OF BLOOD TAKEN FROM GUINEA-PIGS FED BOTULINUM TOXIN

Animal No.	Weight, Gm.	Amount Fed, C c.	Time of Bleeding, Hours	Symptoms	Amount Injected	Toxin
1	950	10	3	0	1 c c. blood	0
			34.5	+	0.5 c c. blood	0
2	530	1	5	0	1 c c. blood	0
			17.5	+	1 c c. blood	0
			17.5	+	1 c c. serum	0
3	675	5	24.8	+	1 c c. blood	+
			24.8	+	1 c c. serum	+
			24.8	+	0.5 c c. serum	S
4*	...	5	30	+	6 c c. blood to guinea-pig	0
5*	...	5	30	+	11 c c. blood to guinea-pig	0
6	525	5	3	0	1 c c. blood	+
			21	+	0.5 c c. serum	+
			21	+	0.1 c c. serum	0
7	425	5	3	0	1 c c. blood	0
			21	0	1 c c. blood	0
			93	+	1 c c. serum	0

* The blood taken from guinea-pigs 4 and 5 was injected into 200 gm. guinea-pigs. S indicates botulism symptoms.

TABLE 4

INJECTION INTO MICE OF BLOOD TAKEN FROM RATS FED BOTULINUM TOXIN

Animal No.	Time of Bleeding in Hours	Amount Injected	Toxin
1	3	1 c c. blood	0
	34.5	0.5 c c. blood	0
2	5	1 c c. blood	0
	17.5	1 c c. blood	0
3	30	2 c c. blood to guinea-pig	0
4	3	1 c c. blood	0
	21	1 c c. blood	0
	93	1 c c. serum	0
5	3	1 c c. blood	0
	21	1 c c. blood	0

The blood from rat 3 was injected intraperitoneally into a 200 gm. guinea-pig. The rats used averaged about 125 gm. in weight. Five c c. were fed in all cases except to no. 2, which was given 1 c c.

with the same amount of toxin. One of them died in a little more than 21 hours, with typical symptoms of botulism, whereas it was a matter of 93 hours before the other animal succumbed.

Toxin in the blood of guinea-pigs is not a constant finding. There seems to be great variability in results when the toxin is given orally. In no case in which the guinea-pigs were fed the toxin did they fail to exhibit symptoms of botulism, but the time of onset of the symptoms was inconstant and variable.

The time intervals for bleeding the rats were the same as those for the guinea-pigs. The rats seemed to be resistant to the toxin throughout the series. There is no evidence in these experiments to show that the toxin gets through their intestines and into the systemic circulation.

PERSISTENCE OF BOTULINUM TOXIN IN THE BLOOD OF GUINEA-PIGS AND RATS GIVEN INTRAVENOUS AND INTRA-MESENTERIC INJECTIONS OF TOXIN

The same toxin as was described in the previous experiments was used in these tests. The intravenous injections were given to guinea-pigs in a superficial vein on the inner aspect of the hind leg, according to Roth's [9] method. The animals were etherized before making the injection. One c c. of toxin was given throughout these experiments.

TABLE 5

Persistence of Botulinum Toxin in the Blood of Guinea-Pigs and Rats Given Intravenous and Intramesenteric Injections of Toxin

Animal No.	Weight, Gm.	Mode of Injection	Toxin in 1 C c. of Blood Injected into Mouse from 3 Hour Bleeding
Guinea-pig 6	475	Intravenous	+
Guinea-pig 18	450	Intravenous	+
Guinea-pig 44	340	Intramesenteric	+
Rat 11	125	Intravenous	+
Rat 19	130	Intramesenteric	+

The rat was etherized and given an intravenous injection into one of the veins in the tail. The intramesenteric injections were made into one of the veins in the mesentery in order to carry the toxin through the portal circulation. After making the injection, a fine silk ligature was placed around the vessel before withdrawing the needle. The needle was withdrawn and the ligature tightened, thus preventing the escape into the peritoneal cavity of any of the toxin. After making the injection, the intestine was replaced and the incision closed.

The animals were bled 3 hours after receiving the injections. Some of them showed marked symptoms of botulism at this time. All of the animals were dead in less than 14 hours, some of them dying in 4 hours. The results are given in table 5.

From the table it may be seen that the toxin was present in the blood of all the animals after 3 hours. No further bleedings were made as many of the animals were in advanced stages of botulism. Quantitative measurements of the titer of the toxin are necessary in order to determine the amount of toxin fixed in the tissues, altered or disposed of in some other way.

[9] Jour. Bacteriol., 1921, 6, p. 249.

SUMMARY

Botulinum toxin when mixed with stomach tissue and abdominal wall tissue from guinea-pigs and mice in vitro and incubated at 35 C. for one hour was not reduced in potency except in two cases in which mouse stomachs were used. The reduction, however, was slight in these cases.

Botulinum toxin was not completely destroyed in the stomachs and small intestines of rats and guinea-pigs receiving injections of the toxin directly into the stomach. Its presence was definitely demonstrated after 12 hours in guinea-pigs and after 3 hours in rats.

In 2 cases out of 7 toxin was demonstrated in the blood of guinea-pigs fed with botulinum toxin. No toxin was found in the blood of rats fed with large doses of botulinum toxin.

Toxin was found in the blood of rats and guinea-pigs 3 hours after intravenous and intramesenteric injections of the toxin.

STUDIES ON RESPIRATORY DISEASES

XXVII. ELECTROPHORETIC POTENTIAL, ACID AND SERUM AGGLUTINATION OF PNEUMOCOCCI *

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In preceding publications from this laboratory we have reported on the electrophoretic potentials on pneumococci of types 1, 2, 3 and 4 and on original and single cell cultures of variants of type 1 produced by Blake. We have also presented data on some relations between these potentials and the virulence of the cultures for the white mouse and for man. For Blake's variants we have confirmed his findings with respect to their virulence, agglutinability, etc., and we have pointed out the parallelism between decreasing virulence, decreasing electrophoretic potential and increasing agglutinability by serum (A, B, C).[1] The experiments reported in this and the succeeding papers were designed to provide further information on the interrelationship of these characteristics of pneumococci.

TECHNIC

The influences of acidity on the spontaneous agglutination and on the electrophoretic potentials of pneumococci were determined by the following technic:

The pneumococcus cultures used in these experiments were of the series described in the preceding publications. J 106, 046, NY III and P_{35} are representative cultures of types 1, 2, 3, and 4, respectively; A, B and C are the variants of type 1 isolated after growth in the presence of homologous antiserum and kindly placed at our disposal by Professor Blake of the Yale School of Medicine. The pneumococcus cultures were grown on blood-agar slants. About 16 hours later, 10 c c. of Blake's broth were poured over each and incubated over night at 37 C. The following morning each of the suspensions was transferred to 300 c c. of 1% glucose broth ($P_H = 7.8$) and were incubated for 24 hours. The bacteria were then sedimented by centrifugalization at 3,000 r. p. m. and were taken up in sterile, distilled water. Their sedimentation and resuspension in water was repeated 3 times. After the third washing, the suspensions were distributed in 0.5 c c. quantities in serologic tubes and in 25 c c. quantities in flasks, the suspensions being of the turbidity desired for agglutination tests. They were then sedimented once more and were held in the ice chest over night. In the morning the supernatant fluid was poured off the sediment

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[1] Falk, Gussin and Jacobson: Jour. Infect. Dis., 1925, 37, p. 481. Falk and Jacobson: Ibid., p. 507.

in each tube or flask, and the sediment was taken up in Northrop-Dekruif buffer at the specified P_H. The bacteria was stirred into uniform suspensions, and were held in a water bath at 37 C. for 1 hour. The larger quantities were used for potential determinations; the smaller quantities were further incubated at room temperature for 24 hours. The extent of agglutination in these suspensions was then determined and recorded. The P_H values were measured colorometrically.[2]

Suspensions of pneumococci were prepared in a similar manner for a series of experiments on the influence of serum on potentials and agglutination. The need for larger quantities of bacteria, however, necessitated the use of larger volumes of broth cultures and their sedimentation in a Sharples supercentrifuge at 30,000 r. p. m. The bacteria were rewashed with 0.85% NaCl solution 3 times and were resuspended in salt solution. Diluted serum and bacteria were mixed in small test tubes and in flasks. The final serum dilutions stated in the tables were calculated to include the dilution by the bacterial suspensions.

THE INFLUENCE OF P_H ON ELECTROPHORETIC POTENTIAL

Measurements of electrophoretic potential were made by the method described in the preceding papers. The results of a series are presented in table 1. The iso-electric points have been determined by graphical interpolation.

TABLE 1

THE INFLUENCE OF PH ON THE ELECTROPHORETIC POTENTIALS OF WASHED PNEUMOCOCCI

+, signifies migration to the cathode; —, to the anode

P_H	Electrophoretic Potentials (in μ/sec) for Cultures						
	A	B	C	J106 1	O46 2	N.Y. 3	P35 4
0	+ 4.7	+ 6.8	+ 5.7	+ 2.6*	+ 1.8	+ 1.9	0†
0.5	+ 6.2	+ 6.8	+ 4.0	+ 7.8	+ 4.2	+ 2.5	+0.8
1.2	+ 5.2	+ 7.4	+ 8.5	+10.0	+ 3.8	+ 4.9	+8.1
1.7	+ 5.0	+ 5.9	+ 6.9	+ 7.7	+ 9.2	+ 3.6	+6.2
2.2	+ 2.8	+ 4.4	+ 6.3	+10.3	+ 0.6	+ 2.8	+5.8
2.8	+ 1.1	— 1.0	+ 1.9	+ 2.2	0	+ 1.2	+4.3
3.0	— 2.1	— 2.5	+ 1.5		— 0.3	+ 1.1	+3.3
3.2	— 5.9	— 6.1	— 5.0				
3.9	— 6.4	— 7.4	— 6.3	— 2.7	— 5.7	— 5.7	—4.3
4.4	— 7.4	— 8.9	— 7.6	— 6.1	— 6.2	—10.6	—3.9
4.7	— 7.4	—10.0	— 8.3	— 5.9	— 6.2	—12.1	—5.0
5.3	— 9.2	—10.6	—12.1	— 7.7	— 6.3	—12.1	—6.5
6.0	— 9.7	—13.1	—14.8	— 9.7	— 7.6	—14.2	—6.5
6.2	—11.7	—14.2	—15.5	—11.3	— 7.6	—14.8	—7.4
6.5	—12.1	—13.1	—17.0	—11.3	— 7.6	—18.9	—8.9

* Some cells moving at a velocity + 8.3.
† Some cells moving at a velocity + 8.9.

It appears from these data: (1) that with increasing acidity, the electrophoretic potentials are reduced beyond $P_H = 6.5$; (2) they reach zero (iso-electric) values in the zone $P_H = 2.7\text{-}3.3$; (3) they become increasingly positive (i. e. the migration is toward the cathode) until maximal values are reached in the zone $P_H = 1\text{-}2.25$; and (4) they

[2] The Northrop-De Kruif buffer was prepared following these authors, Jour. Gen. Physiol., 1922, 4, p. 639.

approach another isopotential point at extreme acidities near $P_H = 0$. It would appear from graphs of these data that the iso-electric points are the following:

For Pneumococcus	PH
A	2.9
B	2.7
C	3.0
1	3.3
2	2.8
3	3.2
4	3.3

We do not consider the differences between these points significantly different from the probable errors of the measurements.

In preceding publications we have pointed out that for pneumococci of the fixed types the usual sequence for decreasing potential (in zones of P_H near neutrality) is: 3, 1, 2, 4, and that it is not changed after washing the organisms. Similarly, the sequence for the variant strains is usually: A, B, C, and it is inverted to C, B, A after washing. The bacteria used in the experiments cited in table 1 show the sequences for washed pneumococci.

THE INFLUENCE OF P_H ON SPONTANEOUS (ACID) AGGLUTINATION OF PNEUMOCOCCI

In table 2 we present the results of a typical experiment showing the zones of acid agglutination.

TABLE 2

THE ACID AGGLUTINATION OF WASHED PNEUMOCOCCI (1 — 4; A, B, C) IN BUFFERED SOLUTIONS

P_H	Agglutination of Culture						
	A	B	C	J106 1	O46 2	N.Y. 3	P35 4
2.4	0	0	++	±	++++	+	+++
3.0	0	++	+++	++	++++	+++	+++
4.4	++	+++	++++	+	++++	±	+++
4.6	++	++	++++	0	++	0	+++
4.9	0	++	+++	0	+	0	+++
5.0	0	0	+++	0	+	0	+++
5.6	0	0	0	0	0	0	+++
6.2	0	0	0	0	0	0	+++
6.4	0	0	0	0	0	0	+++
6.8	0	0	0	0	0	0	+++
7.5	0	0	0	0	0	0	+++
8.0	0	0	0	0	0	0	+++
8.5	0	0	0	0	0	0	+++
9.0	0	0	0	0	0	0	+++
9.5	0	0	0	0	0	0	+++
9.9	0	0	0	0	0	0	+++

0 indicates no agglutination; +, agglutination detectable with 5× lens; ++, agglutination detectable with the naked eye; +++, agglutination complete, supernatant turbid; ++++, agglutination complete, supernatant clear.

It is seen that the sequence of increasing acidity necessary to cause agglutination and the sequence of increasing width of acid agglutinating zone is: A, B, C. This finding may be described in other words, namely: variant C is agglutinated at lower acidity than variant B, and variant B is agglutinated at lower acidity than variant A (original type 1). Also, of the three, A is most easily stabilized by an excessive acidity. The similar sequence for increasing acid agglutinability for cultures of the fixed types is: 3, 1, 2, 4. In each case, the sequences are the same as those for decreasing electrophoretic potential and for decreasing virulence. The cultures used in these experiments had not been washed sufficiently to reverse the potential sequence for the variant strains.

EFFECTS OF ANTIPNEUMOCOCCUS SERUM ON ELECTROPHORETIC POTENTIALS AND ON THE AGGLUTINATION OF PNEUMOCOCCI

In studying the effects of antipneumococcus serum on potentials and agglutination of the variant strains, we have used only a type 1 horse serum. (The variants B and C were derived from a culture of A pneumococci, a type 1 strain.) The results of a typical experiment are cited in table 3.

TABLE 3

EFFECTS OF TYPE 1 ANTIPNEUMOCOCCUS SERUM ON THE ELECTROPHORETIC POTENTIALS (μ/SEC) AND THE AGGLUTINATION OF WASHED SUSPENSIONS OF PNEUMOCOCCUS VARIANTS A, B AND C

Dilution of Serum	A		B		C	
	μ/Sec	Aggn.	μ/Sec	Aggn.	μ/Sec	Aggn.
10	6.8	+++	9.2	++	10.0	++
20	7.7	++++	11.7	++	12.6	+
40	9.4	++++	13.1	0	14.1	0
60	9.4	++	14.1	0	14.8	0
80	9.4	+	....	0	....	0
100	....	0	....	0	....	0
Control	12.6	0	14.8	0	20.6	0

It is seen from the data in table 3 that antipneumococcus serum reduced the potentials on strains A, B and C, the reduction being approximately proportional to the concentration of serum. In all cases the reduction of the potential below approximately 12 μ/sec (circa 15.6 millivolts) is accompanied by agglutination.

In a preceding paper, we have reported that the agglutinability sequence for strains A, B and C was the reverse of the virulence and the potential sequence (A B, C). In table 3, the agglutinability sequence is the same as that for virulence. The explanation for this paradox is

TABLE 4

EFFECTS OF ANTIPNEUMOCOCCUS SERUM ON THE ELECTROPHORETIC POTENTIALS (μ/SEC) AND THE AGGLUTINATION OF WASHED SUSPENSIONS OF PNEUMOCOCCI (TYPES 1-4)

Strain	Type	Serum	μ/Sec								Agglutination							
			Serum Dilutions						Bile	NaCl Control	Serum Dilutions						Bile	NaCl Control
			10	20	40	80	160	320			10	20	40	80	160	320		
J106.....	1	1	6.8	10.0	13.1	14.8	15.4	14.8	12.6	13.1	++++	++++	++++	++	+	0	+*	0
		2	12.1	14.8	....	....	....	....	....	....	0	0	0	0	0	0		0
		3	13.1	15.4	....	....	....	....	....	....	±	0	0	0	0	0		0
O46.....	2	1	11.0	11.3	....	....	....	....	14.8	10.3	+	±	0	0	0	0	+	0
		2	8.5	9.2	11.3	10.3	11.0	....	....	....	+	+	+	+	0	0		0
		3	11.7	11.7	11.7	.. .	....	....	....	....	++	+	+	0	0	0		0
N.Y.....	3	1	13.6	14.8	16.2	16.2	....	....	11.3	16.2	±	0	0	0	0	0	+	0
		2	11.3	14.8	....	....	....	....	....	....	0	0	0	0	0	0		0
		3	12.6	13.6	16.2	17.9	17.0	....	....	....	+++	+++	+++	++	±	0		0
Washington.	4	1	10.0	10.6	12.6	....	....	....	9.7	10.0	0	0	0	0	0	0	+	0
		2	10.3	12.1	13.1	....	....	....	....	....	0	0	0	0	0	0		0
		3	9.7	11.3	13.1	. ..	....	....	....	....	0	0	0	0	0	0		0

* + means dissolution of the pneumococci.

that the pneumococci used in the present experiments had been washed thoroughly and the potential sequence had been reversed. Hence, it is not surprising to find that concurrent with a reversal of the potential sequence there has occurred a reversal of the agglutinability sequence. This observation may also be considered a further confirmation of the view that agglutinability is a function of the potential. In table 4 we present the results of a similar experiment with strains of pneumococci of the fixed types.

It is seen that the type specificity of each serum is as plainly evident in the reductions of electrophoretic potentials as in the agglutination of the bacteria. These results are in harmony with the findings reported by Shibley.[3]

CONCLUSIONS

We have found that for pneumococci, as has already been found for other bacteria, with increasing acidity the potential difference (P. D.) between bacteria and menstruum declines from negative values to an iso-electric point, then to increasing positive values and, near P_H-0. to a second isopotential point.

The iso-electric points of the pneumococci of types 1, 2, 3 and 4 and of variants (B and C) of type 1 (A) are near $P_H = 3$ and are not significantly type specific.

The P. D. for pneumococci is correlated with the inagglutinability by acid. In general, it is found that the higher the P. D. at $P_H = 6.5$, the lower the P_H necessary to cause spontaneous agglutination.

The P. D. values for the variant types can be changed relatively by washing the bacteria. An inversion of the relative potentials on variants A, B and C is accompanied by an inversion in their agglutinability by acid.

Type-specific, homologous and heterologous antipneumococcus serum reduces the negative P. D.; the greater effectiveness of the homologous serum in this regard parallels its greater effectiveness in agglutinating the bacteria.

The correlation between potential and inagglutinability is further evidenced by the inversion of the relative stability of suspensions (A, B, C) when tested with agglutinating serum after they have been washed sufficiently to invert the relative magnitudes of the potentials.

[3] Jour. Exper. Med., 1924, 40, p. 453.

STUDIES ON RESPIRATORY DISEASES

XXVIII. THE ELECTROPHORETIC POTENTIALS, THE DISSOLUTION AND THE SERUM AGGLUTINATION OF PNEUMOCOCCI IN THE PRESENCE OF SODIUM OLEATE *

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Many investigators have observed that the relative solubility of pneumococci by bile appears to be correlated with their virulence for laboratory animals.[1] Hence, we have undertaken a number of experiments on the cytolysis of the pneumococcus variants (A, B and C), which have been described in preceding papers of this series. These strains differ markedly in virulence for mice. We have also included in our experiments, for comparative purposes, representative strains of types 1, 2, 3 and 4.

Falk and Yang [2] have reported that the solubility of pneumococci in sodium oleate solutions parallels their solubility in bile. We have therefore studied the solubility of the pneumococci in solutions of these two reagents. Measurements of the electrophoretic potentials were made on the bacteria in the same suspensions. This was possible because even in a suspension showing "complete" dissolution (as measured macroscopically) a sufficient number of bacteria escape cytolysis to permit of measurements of potential difference (P. D.) by the microscopic method described by Falk, Gussin and Jacobson.[3]

From a series of preliminary tests it appeared that bile and oleate solubility were essentially similar whether conducted in water or in 0.85% NaCl solution, and no significant differences appeared as between strains A, B and C. Furthermore, with 33 and 16% solutions of bile and with a 3.3% solution of sodium oleate the number of washings (1-5) to which the bacteria had been exposed was without effect on the cytolysis. Unwashed pneumococci in broth culture are soluble in bile, but not in oleate. In tables 1 and 2 we present the results of typical

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

[1] Cotoni, Truche and Raphael: The Pneumococcus and Pneumococcal Affections, 1924, p. 40.

[2] Jour. Infect. Dis., 1926, 38, p. 8.

[3] Ibid., 1925, 37, p. 481.

experiments showing the effects of sodium oleate in dissolving pneumococci and in modifying the P. D. between them and their menstruum. We have already presented data in preceding papers to show that bile is practically without effect on the P. D.

TABLE 1

THE DISSOLUTION OF PNEUMOCOCCI (VARIANTS A, B AND C) AND THEIR ELECTROPHORETIC POTENTIALS IN SOLUTIONS OF SODIUM OLEATE

Concentration of Na Oleate, %	Dissolution			P. D. (μ/sec)		
	A	B	C	A	B	C
0	0	0	0	11.9	12.4	16.6
0.0025	0	0	0			
0.005	0	0	0	14.8	12.8	17.4
0.025	+	+	+++			
0.050	+++	++++	+++	14.3	13.1	11.7
0.125	++++	++++	++++	16.4	10.5	15.2
0.25	++++	++++	++++	25.2	25.2	25.2
0.50	++++	++++	++++	29.2	29.2	29.2
1.00	++++	++++	++++	24.2	27.2	26.2
1.25	++++	++++	++++			
2.5	++++	++++	++++	28.4	28.4	28.4
5.0	++++	++++	++++	27.9	27.2	27.2

P_H of all suspensions = 6.4 — 6.8; 0 = no cytolysis; +, ++, +++, ++++ = slight, moderate, extensive and complete cytolysis, respectively.

TABLE 2

THE DISSOLUTION OF PNEUMOCOCCI (TYPES 1, 2, 3 AND 4) AND THEIR ELECTROPHORETIC POTENTIALS IN SOLUTIONS OF SODIUM OLEATE

Concentration of Na Oleate, %	Dissolution				P. D. (μ/sec)			
	J106 1	O46 2	N.Y. 3	P35 4	J106 1	O46 2	N.Y. 3	P35 4
0	0	0	0	0	21.2	20.0	18.4	21.9
0.0025	0	0	0	0	22.7	....	13.9	....
0.005	0	0	0	0	22.7	....	15.5	....
0.025	0	0	0	0	24.3	....	16.6	....
0.050	0	0	+	0	25.2	....	17.9	....
0.125	+++	++	+++	+	24.3	....	....	....
0.25	+++	++	+++	+	24.3	....	....	....
0.50	++	0	++	0	42.5	....	....	....
1.00	+++	++	++++	++	34.0	....	....	....
1.25	+++	++	++++	++	34.0	30.9	....	....
2.5	+++	++	++++	++	29.6	30.9	....	....
5.0	+++	++	++++	+	22.7	30.9	32.4	24.3

From table 1 it appears that there are no significant differences in the Na oleate solubility of the several strains A, B and C. It is clearly evident that the oleate effects significant increases in the P. D. From table 2 it appears further that essentially the same results are obtained with strains of the fixed types.

The possible relation of P. D. to cytolysis warrants a few brief remarks. We have already indicated that pneumococcus dissolution by bile is not associated with an increased P. D., contrary to the finding

with oleate. Hence, it may appear that dissolution and effect on P. D. are not parallel properties of a reagent. It must be recalled, however, that bile is a mixture of many substances, only some of which are known to play a rôle in pneumococcus dissolution. Nonlytic constituents of bile may behave like most substances which have been studied (proteins, peptones, acids, alkalis, salts, etc.) and which have been found to cause reductions in P. D.; hence, the effects of bile on P. D. must be treated with caution. As a rule, the highest potentials on a dispersed particle obtain in the case of suspensions in pure water. The addition of ionizing reagents lowers P. D. The production of an increased P. D. on the addition of such a salt as Na oleate is an unusual phenomenon whose relations to cytolysis may prove to be significant.

TABLE 3

The Effect of Sodium Oleate on the Viscosity of Pneumococcus Suspensions

Suspensions of Pn J 106 (Type 1) in 0.85% NaCl Solution

Concentration of Na Oleate, %	Temperature, C.	η Oleate Solution	η Oleate Solution + Bacteria
0.01	25.0	85.0	85.2
0.10	25.5	90.3	90.8
0.50	25.1	86.8	90.2
1.0	25.0	87.8	89.8
5.0	25.0	90.0	90.8
10.0	25.0	84.1	84.2

These measurements of viscosity were made for us by Mr. R. W. Harrison of our laboratory.

It has been assumed in discussing the data in tables 1 and 2 that the effect of oleate on electrophoretic velocity indicates an effect on electrophoretic potential. That the effect is not on the viscosity of the menstruum and that the assumption is warranted, is indicated from a series of measurements such as those presented in table 3. Although Na oleate produces changes in viscosity, the magnitude of these changes is not proportional to the altered velocity. As the alteration in viscosity is in the direction of an increase, the implication is that the increase in P. D. caused by the oleate is even greater, not smaller, than the measured velocity would signify.

From the discovery that sodium oleate effects an increase in electrophoretic potential, we ventured the prediction that it will increase the stability of pneumococcus suspensions and will reduce the titer of serum agglutinations. In table 4 we present the results of an experiment designed to verify this prediction. The procedure followed was to conduct a series of serum titrations in the presence of various concen-

trations of sodium oleate. Care was taken to distinguish by microscopic examinations between agglutination and the formation of floc from the cytolysate produced by the oleate. It is seen from the data in table 4 that, in suitable dilutions, sodium oleate interferes with the serum

TABLE 4

THE EFFECT OF SODIUM OLEATE ON THE SERUM AGGLUTINATION OF WASHED PNEUMOCOCCI

Strain of Pneumococcus	Concentration of Na Oleate, %	Dilution of Homologous Antiserum							
		6	10	20	40	80	160	320	640
A (type 1)	5.0	*	*	0	0	0	0	0	0
	1.0	*	*	0	0	0	0	0	0
	0.50	*	*	*	*	0	0	0	0
	0.10	+++	++	+++	+	0	0	0	0
	0.05	+++	+++	+++	++	+ 0	0	0	0
	0.01	+++	+++	+++	++	+	0	0	0
	0	+++	+++	++	+	++	0	0	0
B (type 1, variant)	5.0	*	*	*	0	0	0	0	0
	1.0	*	*	0	0	0	0	0	0
	0.50	*	*	*	*	0	0	0	0
	0.10	++	*	*	*	0	0	0	0
	0.05	++	+	+	+	0	0	0	0
	0.01	++	++	++	0	0	0	0	0
	0	+	+	+	+	+	+	0	0
C (type 1, variant)	5.0	*	0	0	0	0	0	0	0
	1.0	*	*	+	0	0	0	0	0
	0.50	*	*	*	0	0	0	0	0
	0.10	+++	+	+	0	0	0	0	0
	0.05	+++	++	++	0	0	0	0	0
	0.01	+++	+++	+++	0	0	0	0	0
	0	+++	+++	+++	+++	+++	+++	+	+
O 46 (type 2)	5.0	+*	*	0	0	0	0	0	0
	1.0	+*	*	0	0	0	0	0	0
	0.50	+*	*	*	0	0	0	0	0
	0.10	+*	*	*	0	0	0	0	0
	0.05	+	0	0	0	0	0	0	0
	0.01	+++	+++	+	0	0	0	0	0
	0	++++	+++	++	++	++	0	0	0
N. Y. (type 3)	5.0	S	0	0	0	0	0	0	0
	1.0	+*	+*	0	0	0	0	0	0
	0.50	+*	+*	+*	0	0	0	0	0
	0.10	++	++	*	*	*	6	0	0
	0.05	+++	+++	+++	0	0	0	0	0
	0.01	+++	+++	+++	++	++	0	0	0
	0	++++	++++	++++	+++	+++	0	+	0

++++; +++; ++; + = agglutination complete; nearly complete; slight; trace; * = formation of nonorganized floc, determined by microscopic examination; S = pneumococci dissolved completely; no floc, no clumps.

agglutination of pneumococci of types 1, 2 and 3, and of variants of type 1—strains B and C.

Mellon, Hastings and Anastasia,[4] following the work of Northrop and DeKruif,[5] have emphasized the significance of the "cohesive factor"

[4] Jour. Immunol., 1924, 9, p. 365.

[5] Jour. Gen. Physiol., 1922, 4, pp. 629, 639, 655; 5, pp. 127, 139; Jour. Exper. Med., 1923, 37, p. 647. Northrop: The Theory and Application of Colloidal Behavior, 1924, 1, p. 70. Green: Jour. Infect. Dis., 1924, 35, p. 5.

in determining the stability of a colloidal suspension. They have reported on the stabilizing effects of sodium oleate and other reagents which are known to affect surface or interfacial tensions. Our experimental findings illustrated in table 4 may be explained along these lines as well as along those indicated above. For reasons which will be presented at length in a later publication we incline to the view that the stabilizing effect of sodium oleate is probably a function of its capacity to increase potential.

SUMMARY

The demonstration by Falk and Yang that pure solutions of sodium oleate dissolve pneumococci as specifically as bile has made possible a comparative study of the effects of oleate on cytolysis, electrophoretic potential and agglutination.

The finding that the variant strains of pneumococci (A, B and C) which show marked differences in virulence for mice are equally soluble in bile or oleate solution is contradictory to the usual statement that virulence and solubility are parallel characteristics.

Bile is practically without effect on electrophoretic potential.

Sodium oleate effects significant increases in the potentials on pneumococci. From this finding it was anticipated that oleate would increase the stability of pneumococcus suspensions and would reduce the titers for serum agglutinations. These anticipations were confirmed by experiment.

These results provide a further confirmation of the view that the agglutinability of pneumococci is correlated with the electrophoretic potential.

NATURE OF THE SOLUBLE PROTEOLYTIC ENZYME OF BACILLUS PROTEUS

STUDIES IN BACTERIAL METABOLISM. LXXVI

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The nature of the products resulting from the action of soluble bacterial enzymes on protein has been a field for speculation for several years. It has been established definitely that at least some of these enzymes hydrolyze proteins of various kinds to molecules of simpler composition, but the character and extent of this digestion does not seem to have been determined. At present, a majority of observers incline to the view that many of these enzymes—as that of Bacillus proteus—bring about deep-seated changes akin to those of tryptic digestion rather than those of the more superficial peptic digestion, but there is little quantitative evidence to support this assumption.

It is somewhat surprising to discover, moreover, that current concepts of peptic and tryptic digestion, as set forth in texts on biologic chemistry, are qualitative rather than quantitative, although there is unmistakable agreement in the main that the former is distinctly less deep-seated in its effects than the latter. It should be remembered, however, that the methods for the assay and quantitative measurement of the products of protein hydrolysis by enzyme action are not well established. Various procedures have been employed, and the wide difference in the choice of reagents, together with some uncertainty of the nature of the actual products measured by these reagents, render direct comparisons between different sets of observations difficult.

Recent studies by Hunter and Smith [1] and by Wasteneys and Barsook [2] working independently, have added much to available knowledge of the quantitative phenomena of peptic and tryptic hydrolysis of protein. These several investigations show quite clearly the difference between the relatively profound cleavage of the protein molecule by trypsin, and the materially lesser cleavage of it by pepsin.

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[1] J. Biol. Chem., 1924, 62, p. 649.
[2] Ibid., pp. 15, 675; 1925, 63, pp. 563, 575.

Wasteneys and Barsook noticed furthermore that after a period of time, there was an apparent decrease in the quantity of products already formed as a result of either tryptic or peptic digestion. This was construed by them to indicate a partial resynthesis of protein. This phenomenon, in other words, is apparently an example of a reversible enzyme action, similar in its general significance to that reported by Kastle and Loevenhart [3] for lipase, and by Hill [4] for maltase.

It is, of course, difficult to predict with much certainty just what will happen in unstable, aqueous solutions of protein and enzymes, especially on long standing, but the figures presented by Wasteneys and Barsook seem convincing that some kind of recompounding has taken place.

It would seem not unreasonable to assume that this compounding process, whatever it is, might also be construed as indicative of a reunion of amino acids, or their complexes, into combinations not hydrolyzable by the enzyme, thus predicating the genesis of a new substance or substances rather than a partial resynthesis of the old. At least, there is no evidence available to make this assumption untenable.

It will be recalled in this connection that Fischer and Abderhalden [5] found that certain peptids resist tryptic digestion, while other peptids, containing the same amino acids, but arranged in different order, are readily hydrolyzed by trypsin. Thus, alanyl-glycine is readily hydrolyzed by trypsin, while glycyl-alanine is refractory. This subject has more than academic interest to biologists.

Returning to bacterial enzymes: Zak [6] has reported that the enzyme of Bacillus pyocyaneus will cause a rather profound qualitative change when it is added to gelatin. Also, and this has been frequently overlooked, a partial resynthesis of the products of digestion seems to have taken place, in his experiments, by the end of the third week of enzyme action, thus bringing at least one microbic enzyme into line with the synthetic action of enzymes of mamalian origin.

The investigation herein reported is a quantitative study of the changes induced in gelatin by the soluble enzyme of Bacillus proteus, by pepsin, and by trypsin respectively. The analytic methods are those previously described.[7] They are based on the excellent procedures of

[3] Am. Chem. J., 1900, 24, p. 491.

[4] J. Chem. Soc., 1903, 83, p. 578.

[5] Ztschr. f. physiol. Chem., 1905, 46, p. 52. Also: Fischer and Bergell: Ber. d. deutsch. chem. Gesellsch., 1903, 36, p. 2592; 1904, 37, p. 3103.

[6] Beitr. z. chem. Physiol. u. Path., 1907, 10, p. 287.

[7] Kendall: J. Infect. Dis., 1922, 30, p. 211.

Folin, modified somewhat to meet the requirements of microbic cultures. It was found that they gave equally precise results in digestates of gelatin by pepsin and trypsin.

PROCEDURE

A 10% solution of gelatin[8] in distilled water was used both for the pepsin and trypsin digestions. One-tenth % of the enzyme[9] by weight was found to be sufficient to induce a characteristic and fairly rapid change in the gelatin. It was necessary to add 1/10% by volume of hydrochloric acid to the gelatin-pepsin

TABLE 1

DIGESTION OF GELATIN: WITH PEPSIN

	Control	1 Day	2 Days	3 Days	4 Days	5 Days	7 Days	8 Days
Total nitrogen.....	1655.4	1655.4	1655.4	1655.4	1655.4	1655.4	1655.4	1655.4
Protein nitrogen.....	1552.4	1483.4	1382.1	1382.1	1319.4	1319.4	1265.6	1261.4
Nonprotein nitrogen	103.0	172.0	273.3	273.3	336.0	336.0	389.8	394.0
Polypeptid nitrogen.	89.7	158.0	258.2	257.9	320.9	319.9	373.5	376.2
Amino nitrogen.....	8.8	9.1	9.5	9.8	9.5	10.5	10.5	11.9
Ammonia nitrogen..	4.5	4.9	5.6	5.6	5.6	5.6	5.8	5.9
Reaction.....	+4.70	+4.75	+4.70	+4.90	+5.05	+5.10	+5.00	+5.10
WITH TRYPSIN								
Total nitrogen.....	1675.5	1675.5	1675.5	1675.5	1675.5	1675.5	1675.5	1675.5
Protein nitrogen.....	1563.5	741.5	705.5	705.5	704.8	698.3	667.5	663.0
Nonprotein nitrogen	112.0	934.0	970.0	970.0	970.7	977.2	1008.0	1012.5
Polypeptid nitrogen.	97.3	905.3	940.6	939.5	939.9	944.6	975.2	980.4
Amino nitrogen.....	9.1	21.7	21.7	22.8	23.1	24.9	24.9	25.2
Ammonia nitrogen..	5.6	7.0	7.7	7.7	7.7	7.7	7.9	6.9
Reaction.....	—9.45	—9.40	—9.50	—9.55	—9.50	—9.60	—9.55	—9.65

WITH BACTERIA-FREE, SOLUBLE ENZYME OF BACILLUS PROTEUS

	Control	4 Day Culture Filtrate	5 Day Culture Filtrate	8 Day Culture Filtrate	14 Day Culture Filtrate
Total nitrogen.....	1.330	1.330	1.330	1.330	1.330
Protein nitrogen.....	1.061	0.389	0.322	0.367	0.413
Nonprotein nitrogen	0.269	0.941	1.008	0.963	0.917
Polypeptid nitrogen.	0.219	0.860	0.916	0.861	0.821
Amino nitrogen.....	0.035	0.065	0.073	0.076	0.067
Ammonia nitrogen..	0.015	0.016	0.019	0.026	0.029

Nitrogen recorded in mg. per 100 cc. culture medium: reaction in cc. of normal acid or alkali per 100 cc. medium, with neutral red as the indicator.

solution, and to add 1% by weight of sodium bicarbonate to the gelatin-trypsin solution. Ten cc. of chloroform were added to the gelatin-bicarbonate-trypsin solution to maintain sterility. This amount did not seem to injure the enzyme action perceptibly.

The gelatin, carefully weighed, was dissolved in 800 cc. of warm (40 C.) distilled water, together with the proper amount of acid or alkali, as the enzyme required. The enzyme, which was obtained as a dry powder, was also carefully weighed and dissolved in 200 cc. of distilled water (temperature below 37 C.)

[8] A good grade suitable for bacteriological use.

[9] A well known commercial preparation.

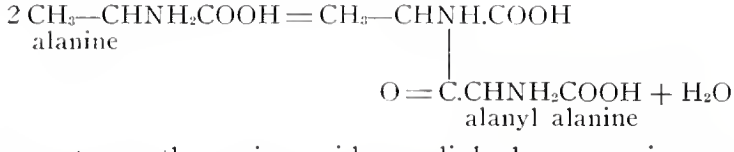
and mixed rapidly with the gelatin. It will be seen that the final volume was 1,000 cc. in each instance.

Control samples were removed in duplicate for the determination of total nitrogen nonprotein nitrogen, amino and ammonia nitrogen, as soon as the digestion mixture was made. They were analyzed at once. The remainder of each gelatin-enzyme solution was placed in the incubator, and kept at 37 C. Samples were taken out for analysis each day at the proper hour for the duration of the test. It should be emphasized that the respective digests were sterile throughout the course of the experiments.

No attempt was made either to measure the speeds of reaction, or to estimate the potency of the respective enzymes used. The principal point at issue was a determination of the type of hydrolysis induced by the soluble enzyme of Bacillus proteus; and the results indicate quite clearly that it is of the tryptic type, although not conforming strictly to the quantitative changes induced by the sample of commercial trypsin with which it is compared.

DISCUSSION

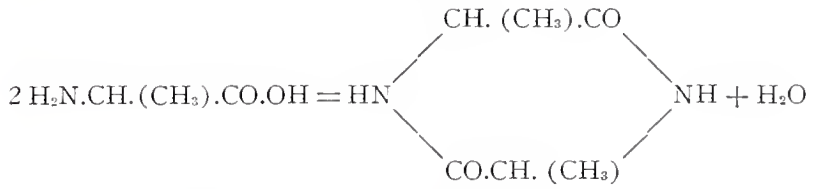
Peptic Digestion.—The most distinctive change observed was a moderately rapid, fairly deep-seated degradation of the gelatin protein from a state where scarcely 7% is not precipitated by the Folin tungstic acid reagent to one where one quarter of the total protein is so altered as to become soluble. This soluble, or nonprotein nitrogen fraction is chiefly "polypeptid nitrogen,"[7] being determinable neither as free ammonia, nor as amino nitrogen by the Sörensen formol titration method. The free ammonia and amino nitrogen increase very little and very slowly with the course of digestion. It might be surmised from the relatively large "polypeptid nitrogen" increase, and the relatively small "amino nitrogen" increase, that the Folin tungstic acid reagent separates proteins from the peptids of relatively complex composition. This, however, is assuming that the amino acids of the protein were united by the protein tie—that simple linkage of the acidic group of one amino acid to the basic group of another: thus,



If, on the contrary, the amino acids are linked as pyrazines—ring compounds containing two nitrogen atoms symmetrically placed in the skeletal compound C_4N_2—a view proposed by Emil Fischer,[10] and elaborated by Fischer and Abderhalden,[11] and others, the facts presented above with respect to the small increase in amino nitrogen, as cleavage

[10] Ber. d. deutsch. chem. Gesellsch., 1905, 38, p. 607.
[11] Ibid., 1906, 39, pp. 753, 2315.

of the protein molecule takes place under the influence of the enzyme, become more readily explained. Thus, according to this theory, alanine condenses to 3, 6 dimethyl—2, 5 dioxo-piperazine, leaving neither a free amino nor a free carboxyl group.



Such a compound would not react in the formol titration.

It might prove profitable, such being the case, to determine the character of the peptids liberated from a variety of proteins by peptic digestion: valuable information might be obtained thus about the peptid complexes and peptid groupings in the various molecules.

Deamination is not a feature in the enzyme cleavage of gelatin, either by pepsin, or trypsin, or by the soluble proteolytic enzyme of Bacillus proteus. Indeed, if such had proved to be the case to any considerable degree, the amino acids would cease to be amino acids, and the theory of protein cleavage by hydrolytic enzymes would have to be recast.

Tryptic Digestion.—Trypsin induced a rapid and apparently deep-seated change in the gelatin protein within 24 hours. The protein insoluble in the Folin reagent decreases from 93% to about 45% during this brief period. There was a slight but unmistakable rise in the amino nitrogen during this interval, amounting to an increase of 12 mg. in 100 cc., or an increase of 150% above the control. This is, however, small in amount when compared with the decided change in the non-protein nitrogen, which was 822 mg., or about 50% of the total nitrogen of the original gelatin. Here, again, it would appear that the Folin reagent either dissolves relatively large molecules, representing, probably, rather large aggregations of amino acids, or the peptids may occur to no inconsiderable degree as piperazines.[10, 11]

Whatever the solubility level of peptids may be in the Folin reagent, it is an extremely precise, quantitative separant for mixtures like the one under discussion.

The free ammonia increased but little as a result of tryptic digestion —about $\frac{1}{10}$%. Here again, determination is not a feature of the enzyme cleavage of protein.

Tryptic digestion is more rapid and more deep-seated than peptic digestion. The reactions of both the peptic and tryptic digestions did not change very much, although in each instance they were toward the acid side. Hydrogen-ion determinations were not made because the "protein error" due to the gelatin was too great to make even reasonably precise comparisons possible.

Digestion of Gelatin with the Soluble Proteolytic Enzyme of Bacillus Proteus.—This enzyme is found in the active state in protein solutions that have been acted upon by Bacillus proteus. It does not appear in an active state in mediums in which utilizable carbohydrate is present.[12] The enzyme solution herein discussed was obtained by filtering gelatin cultures of Bacillus proteus of 4, 5, 8 and 14 days' cultivation respectively through Berkefeld filters, and adding the clear, sterile filtrate to the gelatin medium in the proportion of 5 cc. of the former to 95 cc. of the latter.[13] A control was prepared and analyzed immediately for each day's experiment. This was necessary because the amounts of ammonia and other nitrogen fractions of these enzyme solutions varied with the age of the culture. The enzyme-gelatin mixture was incubated at 37 C. for 3 days in each instance. The solutions remained sterile throughout. This is indicated not only by proper tests for living bacteria but also by the practical absence of ammonia increase, as shown in the table. Deamination proceeds rapidly in sugar-free cultures of Bacillus proteus.[14] The analytical figures show very plainly that sterile solutions containing the soluble enzyme of Bacillus proteus digest gelatin even more rapidly than the commercial trypsin. This is not to be construed as quantitative, however, but merely a qualitative statement. There is no numerical relation inherent in these tables which permits of a direct comparison of the respective amounts of gelatin protein which a unit of pepsin, of trypsin, and of proteus enzyme respectively will render soluble in the Folin precipitant. On the other hand, it is fairly certain that enough of the several enzymes under discussion were present to indicate the general types of change induced by them in the gelatin.

With this explanation clearly in mind, it is quite certain that the changes in the several nitrogen fractions of the gelatin induced by the proteolytic enzyme of Bacillus proteus are almost similar to those

[12] Kendall: Physiological Rev., 1923, 3, p. 438. Kendall and Walker: J. Infect. Dis., 1915, 17, p. 442.

[13] Kendall, Cheatham and Hamilton: J. Infect. Dis., 1922, 30, p. 251.

[14] Kendall and Farmer: J. Biol. Chem., 1912, 12, p. 13.

brought about by trypsin, namely a rapid decrease in the precipitable fraction (protein nitrogen), and the correspondingly large and rapid increase in the fraction soluble in the Folin reagent (nonprotein nitrogen fraction). This in turn is chiefly the "polypeptid nitrogen." The amino and ammonia nitrogen fractions change but little, and slowly.

It would be interesting to compare the peptids that are formed respectively by tryptic and proteus enzyme digestion by accurate chemical analyses. Are they the same? Proteus tryptase is, or may be, active in the human intestinal tract, and it is not a matter of indifference to the host whether the peptids formed by its action are like those during digestion by trypsin, or quite different, and possibly irritating or even poisonous.

SUMMARY

The soluble bacteria-free proteolytic enzyme of Bacillus proteus effects a rapid change in the gelatin molecule. The nature and extent of this change is of the tryptic rather than of the peptic type. The rate and course of action of the proteus enzyme equals, or even exceeds, that of commercial trypsin under parallel conditions, insofar as they are reproduceable in these experiments.

CHEMISTRY OF THE INTESTINAL FLORA OF NURSLINGS

STUDIES IN BACTERIAL METABOLISM. LXXVII

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The intestinal and fecal floras of normal nurslings has been a fruitful field for study in the past, and much information has accumulated about the types of bacteria commonly present, as well as qualitative data concerning the reactions these floras induce in various cultural mediums.[1]

The general conclusions reached in these investigations point uniformly toward the relative absence of proteolytic types among the nursling intestinal bacteria, and the predominance of lactic acid producing microbes, both aerobic and anaerobic, the latter being somewhat more numerous, although difficult to cultivate outside the body.[2]

The prominent specific organisms are Bacillus bifidus, Bacillus acidophilus (several types of each), together with Micrococcus ovalis,[3] and fewer numbers of Bacillus coli and Bacillus aerogenes. The latter belongs to the mucous capsulatus group. Bacillus bifidus is an obligate anaerobe; and both Bacillus acidophilus and Micrococcus ovalis grow somewhat better in initial cultivation outside the body in artificial mediums from which oxygen is excluded. Hence, in making studies of the normal nursling flora for any purpose, anaerobiosis is an indispensible condition, both for specific isolation, and for the study of composite chemical changes induced by these organisms. It follows that intestinal contents should be kept anaerobic from the time the sample is obtained to the final stage of analysis. Cultural mediums, also, should be free from oxygen immediately before inoculation with

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[1] Escherich: Darmbakterien des Säuglings, 1887. Herter and Kendall: J. Biol. Chem., 1908, 5, p. 283; 1910, 7, p. 203. Kendall: Ibid., 1909, 6, p. 257. Schmidt und Strasburger: Die Faeces des Menschen, 1905, Aufl. 2.

[2] Tissier: La Flore Intestinale des Nourissons, 1900. Moro: Wien. klin. Wchnschr., 1900, 13, p. 114.

[3] Thiercelin: Thèse de Paris, 1894.

nursling intestinal bacteria. These precautions are conducive to satisfactory results in all intestinal work involving the combined intestinal flora.

The nitrogenous metabolism of individual normal nursling bacteria —Bacillus bifidus,[4] Bacillus acidophilus,[5] and Micrococcus ovalis [6]—is striking: these several microbes are almost without detectable action on the protein constituents of cultural mediums, with or without carbohydrates. Therefore, the composite chemical picture of microbic activity within the normal alimentary canal would appear to be a continuous and relatively pure lactic acid forming fermentation, with but little, if any, supplemental proteolysis.

On the other hand, the coli-aerogenes type produces moderate changes in the nitrogenous constituents of sugar-free mediums.[7] The addition of utilizable carbohydrates reduces the action of the former on the nitrogenous constituents of cultural mediums to very small amounts.

Although the literature on the qualitative changes induced by these intestinal flora is voluminous, quantitative studies of their composite metabolim do not seem to have been published. Yet such studies are desirable in that they may suggest, or even indicate, just what influence these microbes have on the food in the infantile alimentary tract. Also, some information might confidently be expected about the protective action which the normal nursling types exert in shielding the intestinal tract from alien microbic invaders.

With these several possibilities in mind, a study was made of the metabolism of the composite intestinal flora of 12 nurslings ranging in age from 10 days to 8 months. The babies were normal in every respect, breast fed, and the specimens, together with full data, were obtained through the courtesy of Dr. Charles Burt Reed of Wesley Memorial Hospital. We are deeply indebted to Dr. Reed for his gracious response to our many requests.

The samples were gathered in the simple glass rectal tubes, described several years ago.[8] The cultural mediums were dispensed, with due precautions to remove and exclude oxygen, in anaerobic cultural flasks,[9] which have proved satisfactory for this purpose.

[4] Kendall and Haner: J. Infect. Dis., 1924, 35, p. 77.
[5] Ibid., p. 89.
[6] Ibid., p. 67.
[7] Kendall and Bly: J. Infect. Dis., 1922, 30, p. 239.
[8] Kendall and Walker: Boston M. & S. J., 1911, 164, p. 301.
[9] Kendall, Ryan and Cook: J. Infect. Dis., 1921, 29, p. 227.

TABLE 1

METABOLISM OF BACTERIA OF FECES OF NURSLINGS

Mediums		Control			1 Day			3 Days			6 Days			9 Days		
		Baby 1	Baby 2	Baby 3	Baby 1	Baby 2	Baby 3	Baby 1	Baby 2	Baby 3	Baby 1	Baby 2	Baby 3	Baby 1	Baby 2	Baby 3
Plain Broth	Nitrogen															
	Total	990.0	973.0	973.0	990.0	973.0	973.0	990.0	973.0	973.0	990.0	973.0	973.0	990.0	973.0	973.0
	Protein	810.8	749.0	749.0	810.0	760.2	760.2	788.4	749.0	760.2	777.2	771.4	793.8	766.0	749.0	749.0
	Nonprotein	179.2	224.0	224.0	179.2	212.8	212.8	201.6	224.0	212.8	212.8	201.6	179.2	224.0	224.0	224.0
	Polypeptid	112.0	168.0	168.0	117.6	150.5	151.9	137.2	151.2	128.8	141.4	119.0	62.3	138.6	130.2	89.6
	Amino	47.6	35.0	35.0	37.1	30.8	28.7	36.4	31.5	35.0	39.9	37.1	42.0	42.7	39.9	51.8
	Ammonia	19.6	21.0	21.0	24.5	31.5	32.2	28.0	41.3	49.0	31.5	45.5	74.9	42.7	53.9	82.6
	Reaction	+0.5	+0.7	+0.7	+0.5	+1.0	+1.1	+0.5	+1.4	+1.2	+0.5	+1.5	+0.9	+0.4	+1.8	+0.9
Glucose Broth	Nitrogen															
	Total	990.0	973.0	973.0	990.0	973.0	973.0	990.0	973.0	973.0	990.0	973.0	973.0	990.0	973.0	973.0
	Protein	810.8	771.4	771.4	833.2	771.4	782.6	810.8	771.4	781.4	833.2	782.6	805.0	833.2	771.4	782.6
	Nonprotein	179.2	201.6	201.6	156.8	201.6	190.4	179.2	201.6	191.6	156.8	190.4	168.0	156.8	201.6	190.4
	Polypeptid	126.0	147.0	147.0	102.2	148.4	141.4	123.2	148.4	139.1	100.8	134.4	114.1	95.2	146.3	135.8
	Amino	35.7	33.6	33.6	32.9	27.3	25.2	32.9	28.0	29.4	32.2	32.9	31.5	37.1	33.6	35.0
	Ammonia	17.5	21.0	21.0	21.7	25.9	23.8	23.1	25.2	23.1	23.8	23.1	22.4	24.5	21.7	19.6
	Reaction	+0.5	+1.0	+1.0	+3.3	+5.3	+5.7	+7.8	+9.7	+10.3	+13.8	+9.3	+9.8	+13.7	+9.2	+8.4
Starch Broth	Nitrogen															
	Total	990.0	959.0	959.0	990.0	959.0	959.0	990.0	959.0	959.0	990.0	959.0	959.0	990.0	959.0	959.0
	Protein	810.0	768.6	768.6	822.0	768.6	768.6	810.8	759.4	757.4	810.8	802.2	791.0	766.0	768.6	779.8
	Nonprotein	179.2	190.4	190.4	168.0	190.4	190.4	179.2	201.6	201.6	179.2	156.8	168.0	224.0	190.4	179.2
	Polypeptid	126.0	137.2	137.2	110.6	134.4	140.7	117.6	134.4	137.9	114.8	86.8	91.0	154.0	127.2	114.1
	Amino	35.7	33.6	33.6	36.4	26.6	21.7	38.5	33.6	30.8	37.1	39.2	26.6	32.2	35.7	35.7
	Ammonia	17.5	19.6	19.6	21.0	29.4	28.0	23.1	33.6	32.9	27.3	30.8	50.4	37.8	31.5	29.4
	Reaction	+0.5	+0.8	+0.8	+0.5	+1.3	+1.8	+1.3	+4.1	+6.0	+1.7	+5.6	+4.9	+0.9	+5.2	+5.2
Milk	Nitrogen															
	Total	514.0	511.0	511.0	514.0	511.0	511.0	514.0	511.0	511.0	514.0	511.0	511.0	514.0	511.0	511.0
	Protein	481.6	432.6	432.6	481.6	443.8	443.8	481.6	443.8	443.8	470.4	477.4	466.2	459.2	455.0	477.4
	Nonprotein	32.4	78.4	78.4	32.4	67.2	67.2	32.4	67.2	67.2	43.6	33.6	44.8	54.8	56.0	33.6
	Polypeptid	8.6	56.0	56.0	7.2	46.2	46.2	7.2	43.4	23.8	18.4	11.2	19.6	28.2	30.8	11.2
	Amino	21.7	14.0	14.0	21.7	12.6	14.0	21.0	14.7	14.0	21.0	17.5	19.6	21.0	18.6	17.5
	Ammonia	2.1	8.4	8.4	3.5	8.4	7.0	4.2	8.1	8.4	4.2	4.9	5.6	5.6	5.6	4.9
	Reaction	+1.8	+1.8	+1.8	+1.8	+4.4	+4.3	+3.1	+9.1	+2.1	+8.8	+5.0	+10.8	+15.3	+8.0	+10.9

Nitrogen is recorded in mg. per 100 cc. culture medium; reaction in cc. normal acid or alkali per 100 cc. culture medium, using neutral red as an indicator.

The mixed samples of intestinal bacteria were inoculated into cultural mediums, within half an hour of the time they were obtained from the infant, using 1 Gm. of fecal mass to 20 cc. of nutrient, oxygen-free broth as the inoculum. One-half cc. of the mixture thus diluted was added to each flask.

In the earlier studies several mediums were used, including besides plain, glucose and starch broths, and milk—lactose, saccharose and manitol enriched mediums. The latter furnished no information not obtainable in the first four mentioned, and were soon discarded. Gelatin, 5%, was added to each fermentation medium to enrich the nitrogenous constituents. Several flasks of each kind of medium were inoculated, so that a 1, 3, 6, and 9 day examination, each of a separate culture, could be made. The chemical methods employed have been described previously;[10] they were followed exactly in their entirety. They included the determination of total, nonprotein, amino and ammonia nitrogen, and the reaction in terms of titrable acidity.[11] Gram stains were made from the several flasks as they were removed from the incubator for analyses; also, similar stains were made from the original inoculum.

It will be noted that these several mediums—plain, glucose and starch-gelatin broths, and milk—provide different diets for the mixed intestinal flora with which they are inoculated. Some information, therefore, should be elicited, outside the intestinal environment, to be sure, of the gross effects of various foods on the chemistry of these microbes.

The subjoined tables contain representative analyses of the nitrogenous changes induced in the plain gelatin broth, the dextrose and starch-gelatin broth, and milk by the mixed intestinal floras of 3 of the 12 babies studied. Of these, number 1 is from a 10-day baby; number 2 is from a baby 6 months old, and number 3 is from a baby 8 months old. All were breast fed and normal in every respect.

DISCUSSION

The cultural, morphologic and chemical studies of these several mixed intestinal floras indicate that there is some disproportionate development of bacteria of the colon type relative to the bifidus type. This is most pronounced in plain gelatin broth, where there is no carbo-

[10] Kendall: J. Infect. Dis., 1922, 30, p. 211.

[11] Hydrogen ion determinations were not made because the gelatin (5%) added to the several broth mediums introduced a "protein error" that interfered with the colorimetric determinations.

hydrate present. This might confidently be expected, as members of the bifidus-acidophilus group grow poorly in mediums from which utilizable carbohydrate is excluded. The amount of protein utilized for energy, however—greatest in number 3—is comparatively little, even in sugar-free mediums, amounting to but 61.6 mg. on the 9th day. It is silightly greater than that produced by pure cultures of Bacillus coli under similar conditions.[7] Twenty-seven milligrams, or about 40% of this increase, occurred during the last 3 days of incubation, suggesting that a relatively small number of proteolytic organisms were present in the original sample, but under the favorable conditions created by the absence of fermentation acids, they multiplied rapidly after the first week. This is commonly observed in specimens from normal nurslings. A similar phenomenon is encountered whenever nursling's feces are exposed to the air for a short time before inoculation. This disproportion between bifidus-acidophilus types and the more putrefactive organisms is less conspicuously shown in the starch mediums, and is nearly absent in glucose gelatin mediums, and in milk. In the glucose and milk mediums, the striking change is in the reaction, which becomes very acid. There is evidence that some of the nonprotein nitrogen is changed to protein nitrogen through incorporation into the substance of some of the newly formed bacteria. Of course, the difference between the protein and the nonprotein nitrogen is not wholly discernible as a formation of bacterial protein, because some of the latter is also used for nonstructural purposes, although apparently not for energy.

SUMMARY

The nitrogenous metabolism of the mixed intestinal flora of a series of 12 normal nurslings shows that the nitrogenous changes induced by the meirobes are relatively little. Proteolysis of bacterial origin is minimal.

The striking change induced by the mixed flora is a pronounced fermentation of utilizable carbohydrates, particularly glucose and lactose. In these mediums, there is a rapid and intense formation of acid, with almost no indication of nitrogenous change in the constituents of the medium other than those ultimately associated with the anabolic phase of bacterial activity.

This pronounced fermentation (principally lactic) is characteristic of the chemistry of the combined fecal bacteria of nurslings.

CHEMISTRY OF THE INTESTINAL BACTERIA OF ARTIFICIALLY FED INFANTS

STUDIES IN BACTERIAL METABOLISM. LXXVIII

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That period in the life of a young child when its dietary needs exceed the ability of the mother to provide, and when therefore supplemental food is required, is one of transition, during which the young person is deprived gradually of the sterile, exquisitely compounded maternal milk, and provided, in accordance with its needs, with pabulum of considerably different character and composition.

One of the seemingly inevitable accompaniments of this change in regimen is a corresponding readjustment of the types and chemical activities of bacteria in the adolescent intestinal tract.

The principal chemical changes in the food of the young person are: first, a reduction in the lactose, which forms a relatively large proportion of the energy-containing substance of mother's milk; second, an increase in the protein; and finally, a partial substitution of sucrose, starch or other carbohydrate for the milk sugar. Also, the new food is far richer in bacteria than the breast milk.

The relatively large variety of bacteria that are swallowed with the artificial food are not reflected, as a rule, in the normal resident flora of the alimentary canal; indeed, comparatively few types become colonized in the intestinal tract.

This change in regimen and the microbic response thereto leads to new conditions within the immature digestive tract which call on latent untried defenses of the body.

Prominent among these conditions is the alteration in the carbohydrate-protein ratio in the food, the latter being disproportionately increased. This leads to the accumulation of a protein residuum in the large intestine, frequently not accompanied by carbohydrate in any form. Coincidentally, the obligately corbohydrophilic microbes, Bacillus bifidus and Bacillus acidophilus, are gradually supplanted by less fas-

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tidious organisms, usually of the colon type, together with smaller numbers of bacteria of the mesentericus group and others.

It should be remembered in this connection that the individual components of the intestinal flora are not fixed and inviolate even under normal conditions: for unknown reasons, they vary somewhat from season to season. The seasonal shifting of some of the intestinal microbes is usually more marked in normal young persons than in normal adults: also, the resident microbes are rather more active, possibly due to the notorious irregularity of adolescent dietary habits. This leads to greater variation and greater plasticity in the percentage composition of the intestinal flora.

The alteration in flora from a predominance of lactic acid bacteria of the bifidus-acidophilus type to the more versatile colon type is initiated by the shifting of the carbohydrate-protein ratio just referred to. The colon group is adaptive in its energy relatoins to carbohydrate and to protein. From the former, lactic acid is formed; from the latter, indol and other putrefactive products. Some, at least, of these aromatic putrefactive products, as indol, are absorbed from the alimentary canal, carried in the blood stream to the liver, and under normal conditions and in normal amounts, oxidized and paired (detoxicated) before they are finally excreted in the urine.[1] It is worthy of note that these substances are rarely, if ever, found either in the intestinal contents or the feces of normal nurslings.

It follows that the appearance of these aromatic putrefactive substances, associated both with the change in regimen from breast milk to artificial food, and the concomitant appearance of colon and related organisms, leads to the functioning of the oxidizing and pairing capacities of the liver in the young adolescents. Obviously this function is not called on in the absece of aromatic putrefactive products, as indol, in the period of normal lactic activity throughout the intestinal tract, that is, during the period of full breast milk feeding. The suppression of these putrefactive products even when colon bacilli are prominent in the intestinal flora is readily accomplished by providing the organisms continuously with utilizable carbohydrate, as glucose or lactose. The bacilli are lactic acid producing bacilli under these conditions. This is shown in the tables that follow.

A study was mode of the chemistry of the mixed flora of 10 young children, ranging in age from 10 months to 2 years Clinically, these

[1] Kendall: Physiol. Rev., 1923, 3, p. 438.

children were normal; no pronounced disturbances, digestive or otherwise, were suspected. Two of these had persistent green stools, not diarrheal, however. The diets were somewhat diversified, both qualitatively and quantitatively, in accordance with the age. Cow's milk, however, was the prominent food in each case.

The 3 cases selected for discussion here were chosen because they represent the extremes as well as the mean of the chemistry met with in the group of artificially fed infants. Number 1 of the series herein reported was a child 10 months old, having a normal intestinal history. Artificial feeding began about the eighth month. The particular sample of feces studied was soft, dark yellow and of a firm consistency. Number 2 was a child 20 months of age, normal in every respect. Number 3 was about 14 months old. The feces were rather dark, somewhat tarry, but otherwise normal.

The fecal material was gathered in the rectal tubes described in the previous study, with precautions to retain anaerobiosis. The methods employed were those already discussed,[2] they were followed in their entirety.

DISCUSSION

The most conspicuous feature of the chemistry of the three specimens herein tabulated is the marked difference in the nitrogenous changes induced respectively in the plain gelatin broth cultures and in the glucose broth cultures. In the former there is unequivocal evidence both of considerable cleavage of the protein molecule, shown both in the increase of the nonprotein fraction, and by the utilization of considerable amounts of this protein derivative for energy, indicated by the increase in ammonia (deamination). The former amounts to from 10 to more than 20% of the total nitrogen of the medium; the latter, from a minimum of 51 mg. to a maximum of 116 mg.

The glucose gelatin broth medium, on the other hand, which differs merely in that 1% of glucose is added, shows a very moderate change in the nonprotein nitrogen fraction in numbers 1 and 3; number 2 is rather unusual in that nearly as much change occurs in the protein fraction in the glucose medium as in the plain sugar-free fraction. This phenomenon is not accidental, because it is repeated almost quantitatively in the starch gelatin medium. It predicates a nonglucose-fermenting microbe in the intestinal flora. The ammonia increase in the glucose mediums is very little indeed, amounting to from 1 to 4 mg.,

[2] Studies in Bacterial Metabolism LXXVII, J. Infect. Dis., 1926, 38, p. 200.

TABLE 1

METABOLISM OF BACTERIA OF FECES OF BOTTLE FED INFANTS

Mediums		Control			1 Day			3 Days			6 Days			9 Days		
		Infant 1	Infant 2	Infant 3	Infant 1	Infant 2	Infant 3	Infant 1	Infant 2	Infant 3	Infant 1	Infant 2	Infant 3	Infant 1	Infant 2	Infant 3
Plain Broth	Nitrogen															
	Total	1071.0	938.0	987.0	1071.0	938.0	987.0	1071.0	938.0	987.0	1071.0	938.0	987.0	1071.0	938.0	987.0
	Protein	824.6	882.0	740.6	813.4	736.4	763.0	746.2	792.4	550.2	723.8	736.4	527.8	723.8	714.0	494.2
	Nonprotein	246.4	56.0	246.4	257.6	201.6	224.0	324.8	145.6	436.8	347.2	201.6	459.2	347.2	224.0	492.8
	Polypeptid	195.3	4.2	191.1	180.0	137.2	142.8	229.6	50.4	259.0	191.8	96.6	260.4	176.4	105.0	284.0
	Amino	35.7	36.4	33.6	42.0	39.2	42.7	21.7	39.2	98.7	23.8	38.5	114.8	30.9	58.1	119.9
	Ammonia	15.4	15.4	21.7	35.0	25.2	38.5	95.2	56.0	79.1	131.6	66.5	84.0	139.9	60.9	88.9
	Reaction	+0.3	+0.2	+0.4	+1.2	+0.5	+2.6	+1.4	+0.7	+2.4	+1.4	+0.8	+1.8	+1.2	+0.8	+1.3
Glucose Broth	Nitrogen															
	Total	1071.0	938.0	987.0	1071.0	938.0	987.0	1071.0	938.0	987.0	1071.0	938.0	987.0	1071.0	938.0	987.0
	Protein	824.6	882.0	751.8	835.8	848.4	852.6	813.4	781.2	769.2	813.4	781.2	763.0	824.6	792.4	751.8
	Nonprotein	246.4	56.0	235.2	235.2	88.6	134.4	257.6	156.8	217.8	257.6	156.8	224.0	246.4	145.6	235.2
	Polypeptid	195.3	5.6	186.9	180.6	36.8	81.2	197.4	105.0	161.8	197.4	96.6	168.0	151.2	84.0	179.2
	Amino	35.7	35.0	26.6	37.8	35.0	30.8	34.1	33.6	32.9	34.1	40.6	32.2	44.8	42.0	32.2
	Ammonia	15.4	15.4	21.7	16.8	16.8	22.4	16.1	18.2	23.1	16.1	19.6	23.8	25.2	19.6	23.8
	Reaction	+0.7	+0.8	+0.7	+3.8	+3.3	+4.8	+9.3	+8.0	+8.4	+9.1	+9.0	+10.1	+8.1	+8.8	+10.4
Starch Broth	Nitrogen															
	Total	1029.0	903.0	903.0	1029.0	903.0	903.0	1029.0	903.0	903.0	1029.0	903.0	903.0	1029.0	903.0	.....
	Protein	805.0	858.2	679.0	793.8	746.2	656.6	737.8	701.4	376.6	771.4	723.8	421.4	782.6	723.8	.....
	Nonprotein	224.0	44.8	224.0	235.2	156.8	246.4	291.2	201.6	526.4	257.6	179.2	481.6	246.4	179.2	.....
	Polypeptid	195.0	1.4	176.4	169.4	78.4	154.0	221.2	126.0	351.4	183.4	99.4	266.0	184.8	100.8	.....
	Amino	33.6	29.4	28.7	34.5	46.9	53.9	42.0	41.3	86.8	37.8	43.4	96.6	44.8	41.3	.....
	Ammonia	15.4	14.0	18.9	25.9	31.5	38.5	28.0	34.3	88.2	36.4	36.4	119.0	16.8	37.1	.....
	Reaction	+0.3	+0.2	+1.4	+6.0	+2.9	+3.6	+7.5	+6.5	+4.9	+7.0	+7.3	+3.3	+8.8	+7.9	.....
Milk	Nitrogen															
	Total	518.0	511.0	462.0	518.0	511.0	462.0	518.0	511.0	462.0	518.0	511.0	462.0	518.0	511.0	.....
	Protein	495.6	471.8	394.8	484.4	471.8	417.2	484.4	460.6	406.0	495.6	460.6	383.6	495.6	471.8	.....
	Nonprotein	22.4	39.2	67.2	33.6	39.2	44.8	33.6	50.4	56.0	22.4	50.4	78.4	22.4	39.2	.....
	Polypeptid	0.0	16.8	46.2	9.8	16.8	23.1	9.8	28.0	33.6	0.0	28.0	56.0	—1.4	16.8	.....
	Amino	18.9	18.2	12.6	20.3	16.8	16.1	20.3	18.9	16.1	18.9	17.5	16.1	21.0	18.2	.....
	Ammonia	3.5	4.2	8.4	3.5	5.6	5.6	3.5	3.5	6.3	3.5	4.9	6.3	2.8	4.2	.....
	Reaction	+1.7	+1.5	+1.6	+8.4	+3.5	+6.9	+11.1	+11.7	+13.5	+12.0	+12.8	+15.6	+12.4	+14.5	.....

Nitrogen recorded in mg. per 100 cc. culture medium; reaction in cc. normal acid or alkali per 100 cc. culture medium, using neutral red as an indicator.

respectively. This is merely another striking instance of the sparing action of carbohydrate for protein.

The starch gelatin mediums are much like the glucose gelatin mediums, except that the ammonia production is somewhat greater in specimens 1 and 2. Specimen 3 is interesting in that the starch and plain gelatin nitrogenous changes are of like mangnitudes, indicating the absence of considerable numbers of starch fermenting bacteria in the intestinal flora. It is not uncommon to encounter intestinal contents, particularly in young children, which do not contain active starch fermenting bacteria.

Milk cultures were interesting and significant. Practically no change was detectable among the nitrogenous constituents of this medium, as is shown clearly in the tables. The nitrogenous changes induced by the organisms in the milk cultures are quantitatively almost identical with those brought about by bacteria of the normal nursling alimentary canal.[3] The milk cultures, in other words, are pure or almost pure lactic acid fermentations. One factor that may be of some importance in determining the growth of bacteria in milk is the relatively great amount of acid that is generated in this medium, even from the earliest hours of incubation. It is certain that many of the proteolytic types are restrained in their development in a rapidly increasing acid reaction, and the comparative freedom of these milk mediums from proteolytes would seem to bear out the correctness of this assertion.

CONCLUSIONS

The analyses of the composite changes induced in suitable cultural mediums by the intestinal floras of artificially fed infants reported herewith are representative for the small series studied. They do not necessarily typify those that might be encountered in other similar groups. Hence, they are to be considered as suggestive rather than definitive.

The outstanding feature centers around the striking difference between the nitrogenous metabolism of the floras in plain sugar-free mediums and in carbohydrate-enriched mediums, respectively.

In the former, the nitrogenous constituents are rather profoundly degraded, and the microbes utilize the products of degradation—polypeptids apparently—for energy, as well as for structure.

The addition of utilizable carbohydrate to such mediums, prior to the infection with the intestinal organisms, shifts the catabolism of the

[3] Kendall and Haner: J. Infect. Dis., 1924, 35, pp. 67, 77, 89.

bacteria from protein to carbohydrate. This is indicated both by the very slight changes detectable in the nitrogenous constituents of the carbohydrate-containing mediums, and also by the rapid accumulation of acid. Stated differently, the intestinal floras of artificially fed children induce putrefactive changes in sugar-free mediums, and fermentative changes in the same mediums to which carbohydrates have been added.

It must be remembered that the effects of supplying utilizable sugars to the intestinal organisms, which are so strikingly brought out in the tables, are those induced in test tubes, outside the intestinal environment. It is very probable, however, that parallel changes would take place within the alimentary canal, provided utilizable carbohydrate could be kept continuously at levels of the intestinal tract where proteolysis would otherwise take place.

CHEMISTRY OF THE INTESTINAL FLORA OF NORMAL ADULTS

STUDIES IN BACTERIAL METABOLISM. LXXIX

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A normal healthy adult, enjoying a liberal mixed diet, undisturbed by obstinate constipation, excretes daily a mass of bacteria from the intestinal tract that has rather conservatively been estimated to contain some 30 trillion organisms.[1] For comparison, this prodigious number is of the order of the number of seconds in a million years.

This excretion of microbes goes on for years, under normal conditions, without apparent effect on the host. This does not preclude the possibility of slowly cumulative effects, which may occur without apparent connection with the intestinal flora.

It is probable, also, that the intestinal flora varies somewhat from time to time in respect to the relative proportions of types, numbers, and perhaps most important of all, their products of metabolism. Also, the season of the year (in temperate zones the range is 100 degrees F.), the occupation of the individual, his dietary likes and dislikes, together with seasonal variations in the kinds of bacteria that are ingested with the food, are among the more important external factors that influence the intestinal flora, and to these in turn should be added the potency of digestive enzymes, both gastric and intestinal, and accessory intestinal secretions, gastric and intestinal mobility, and the desiccating power of the large intestine. Yet with all these variables, the intestinal flora as a whole seems to remain singularly constant over considerable periods of time, so far as available information shows, due, in part no doubt, to the peculiar adaptability of the comparatively few kinds of habitual intestinal microbes to the alimentary environment. Stated differently, many adventitious microbes, including some, as Bacillus bulgaricus, that are deliberately administered,[2] fail to acclimatize themselves in the intes-

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[1] Kendall: J. Med. Res., 1911, 25, p. 135.
[2] Herter and Kendall: J. Biol. Chem., 1908, 5, p. 293.

tinal tract, even when introduced under favorable dietary conditions, in large numbers.

The question has often been raised—what is the effect of the intestinal flora on the nutritives in the alimentary canal? How much chemical change is induced by the intestinal microbes in the intestinal contents from the duodenum to the sigmoid? This cannot be answered for obvious reasons. Indirect information, however, of considerable value can be obtained from studies of cultures in artificial mediums.

The metabolism of important individual members of the intestinal flora has been investigated, and qualitative observations have been made on the changes induced in cultivation outside the body by mixed flora in various mediums, such as gas formation, proteolytic activity and the production of aromatic products resulting from the microbic decomposition of proteins and protein derivatives.

There seems to be no quantitative measure of the nature and extent of nitrogenous metabolism of the composite intestinal flora reported, however, up to the present time. It must be emphasized in this connection that the chemical changes induced by the fecal flora do not necessarily indicate the chemistry of alimentary microbes at the upper levels of the small intestine. Nevertheless, the fecal flora contains representatives of the entire alimentary microbic population, and the effects of the combined microbic spectrum on various cultural mediums are of significance in the interpretation of alimentary conditions.

The following tables contain analytical data obtained by inoculating the intestinal flora obtained from normal adults into a variety of cultural mediums, using the methods and procedure outlined in study LXXVII of this series, and studying the changes induced at varying intervals.

Eight persons were studied, and one to several examinations were made of each at irregular intervals. Some attention was paid to the dietary history of each subject. None was suffering from chronic constipation or other noteworthy alimentary disturbance.

The samples were freshly obtained, kept anaerobic, and inoculated as soon as possible into the several mediums described previouly,[3] first freeing them from dissolved oxygen. Suitable controls were utilized throughout. The analytical details of 3 representative samples appear in the subjoined tables.

[3] Study LXXVII, this series.

TABLE 1

METABOLISM OF BACTERIA OF FECES OF NORMAL ADULTS

Mediums		Control			1 Day			3 Days			6 Days			9 Days		
		Case 1	Case 2	Case 3	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3
Plain Broth	Nitrogen															
	Total	980.0	938.0	980.0	980.0	938.0	980.0	980.0	938.0	980.0	980.0	938.0	980.0	980.0	938.0	980.0
	Protein	800.8	882.0	800.8	767.2	747.6	789.6	778.4	736.4	778.4	778.4	814.8	778.4	778.4	747.6	767.2
	Nonprotein	179.2	56.0	179.2	212.8	190.4	190.4	201.6	201.6	201.6	201.6	123.2	201.6	201.6	190.4	212.8
	Polypeptid	128.8	4.2	128.8	141.2	131.6	128.8	100.8	133.0	124.6	79.8	46.2	81.2	51.8	114.8	70.0
	Amino	29.4	36.4	29.4	35.2	38.5	34.3	21.7	37.1	29.4	22.4	35.0	30.8	10.5	33.6	35.0
	Ammonia	21.0	15.4	21.0	36.4	20.3	27.3	79.1	31.5	47.6	99.4	42.0	89.6	139.3	42.0	107.8
	Reaction	+0.4	+0.2	+0.4	+1.2	+0.5	+0.6	+0.9	+1.4	+1.5	+1.3	+1.4	+0.9	+1.6	+1.6	+0.9
Glucose Broth	Nitrogen															
	Total	980.0	938.0	980.0	980.0	938.0	980.0	980.0	938.0	980.0	980.0	938.0	980.0	980.0	938.0	980.0
	Protein	812.0	882.0	812.0	800.8	848.4	800.8	812.0	803.6	800.8	812.0	803.6	812.0	823.2	803.6	812.0
	Nonprotein	168.0	56.0	168.0	179.2	89.6	179.2	168.0	134.4	179.2	168.0	134.4	168.0	156.8	134.4	168.0
	Polypeptid	119.0	5.6	119.0	128.8	37.8	126.7	117.6	79.8	124.6	116.2	77.7	110.6	103.6	78.4	109.2
	Amino	29.4	35.0	29.4	27.3	35.0	29.4	26.6	36.4	29.4	30.8	37.8	31.5	30.1	35.7	32.9
	Ammonia	19.6	15.4	19.6	23.1	16.8	23.1	23.8	18.2	25.2	21.0	18.9	25.9	23.1	20.3	25.9
	Reaction	+0.6	+0.8	+0.6	+7.2	+3.1	+3.6	+8.5	+7.9	+5.5	+8.5	+9.0	+7.6	+10.0	+9.3	+6.0
Starch Broth	Nitrogen															
	Total	945.0	903.0	945.0	945.0	903.0	945.0	945.0	903.0	945.0	945.0	903.0	945.0	945.0	903.0	945.0
	Protein	777.0	858.2	777.0	788.2	722.6	765.8	788.2	722.6	732.2	788.2	757.4	732.2	788.2	735.0	743.4
	Nonprotein	168.0	44.8	168.0	156.8	180.4	179.2	156.8	180.4	212.8	156.8	145.6	212.8	156.8	168.0	201.6
	Polypeptid	121.8	1.4	121.8	103.6	127.2	127.4	98.0	123.9	152.6	95.2	88.9	144.2	72.8	99.4	133.0
	Amino	27.3	29.8	27.3	25.2	33.6	26.6	28.0	33.6	30.8	28.0	32.9	35.7	36.4	34.3	35.7
	Ammonia	18.9	14.0	18.9	28.0	19.6	25.2	30.8	23.1	29.4	33.6	23.8	32.9	47.6	34.3	32.9
	Reaction	+0.4	+0.2	+0.4	+2.4	+1.0	+2.4	+2.8	+7.4	+4.5	+7.8	+8.3	+5.3	+6.5	+7.3	+5.6
Milk	Nitrogen															
	Total	497.0	504.0	497.0	497.0	504.0	497.0	497.0	504.0	497.0	497.0	504.0	497.0	497.0	504.0	497.0
	Protein	441.0	471.8	441.0	429.8	471.8	429.8	429.8	460.6	429.8	429.8	462.6	429.8	441.0	460.6	429.8
	Nonprotein	56.0	32.2	56.0	67.2	32.2	67.2	67.2	43.4	67.2	67.2	41.4	67.2	56.0	43.4	67.2
	Polypeptid	32.2	9.8	32.2	42.0	9.8	42.0	36.4	21.0	36.4	40.6	20.4	33.6	26.6	19.6	35.0
	Amino	14.0	18.2	14.0	13.3	18.9	14.0	18.2	18.9	18.9	15.4	16.8	19.6	18.2	20.3	17.5
	Ammonia	9.8	4.2	9.8	11.9	3.5	11.2	12.6	3.5	11.9	11.2	4.2	14.0	11.2	3.5	14.7
	Reaction	+1.6	+1.5	+1.6	+4.8	+2.6	+4.3	+12.5	+11.0	+7.9	+16.5	+15.0	+9.0	+17.3	+18.0	+10.1

Nitrogen recorded in mg. per 100 cc. culture medium; reaction in cc. normal acid or alkali per 100 cc. culture medium, using neutral red as an indicator.

DISCUSSION

The general trend of the changes in the nitrogenous constituents of the several mediums are clearly defined by the sixth day of incubation. Beyond that time their quantitative significance decreases, at least in relation to probable parallelism in the intestinal tract.

The outstanding feature of the plain gelatin broth cultures is the comparative absence of evidences of enzymic action on the protein. The protein nitrogen fraction does decrease somewhat—most in number 2, and less in numbers 1 and 3—but in no instance is the decrease in the protein nitrogen suggestive of the presence of hydrolytic cleavage such as might be confidently expected if organisms of the proteus type were numerous or active.[4] On the other hand, the increase in ammonia nitrogen is very striking. It is most marked in cultures of 1 and 3, where it amounts to about 100 mg. on the ninth day. This is about 11% of the total nitrogen of the medium. The bacteria that are most active in the plain gelatin medium are therefore either unusually active deaminizers, or more probably, unusually active growers, because the polypeptid nitrogen fraction decreases rapidly. This fraction seems to be peculiarly involved, as a source of energy for most microbes, in sugar-free mediums.

On the other hand, in mediums containing glucose, but otherwise of the same composition, the polypeptid nitrogen changes very little relatively. Also, the ammonia fraction increases very slowly, indicating clearly that little deamination occurs when utilizable carbohydrate is available for energy in place of protein. This is equivalent to a measure of the sparing action of utilizable carbohydrate for protein. Coincident with the growth of bacteria in the glucose gelatin, there is a rapid increase in the titrable acidity.

The changes in the starch gelatin medium are reminiscent of the plain gelatin in respect to the change in the protein nitrogen fraction, but quantitatively much less with respect to the ammonia nitrogen fraction. This suggests that a much smaller proportion of the composite intestinal flora utilizes starch for energy than glucose for energy. This may have some theoretical importance in determining the effects of abundant starch feeding on the intestinal flora, particularly in cases of deficient amylase secretion. Certain bacteria, as the gas bacillus (Bacillus welchii), can increase to abnormal proportions under such conditions, leading to ill defined, but significant symptomatology.

[4] Kendall, Cheetham and Hamilton: J. Infect. Dis., 1922, 30, p. 251.

Milk cultures undergo little change in their nitrogenous constituents even after prolonged incubation. The striking feature is the development of progressive acidity, which becomes relatively great even after 6 days' incubation. This rapid development of an acid reaction restricts or restrains the more proteolytic types of the intestinal flora, and also certain types of the fermentative microbes, as the gas bacillus, and most members of the mucosus capsulatus group.

The chemistry induced in milk by the mixed flora of these normal adults is quantitatively very much like that induced by the normal nursling bacteria under the same circumstances. Qualitatively, however, the organisms concerned are dissimilar. Bacillus bifidus and, usually, Bacillus acidophilus are not found in noteworthy numbers among the adult intestinal types. Nevertheless, the striking similarity observed in the chemistry of the milk cultures throughout this series (LXXVII, LXXVIII, LXXX) has much more than academic interest in the study of intestinal bacteria in relation to intestinal conditions.

CONCLUSIONS

The general results obtained from the metabolic study of these fairly representative intestinal floras from normal adults is a chemical picture of rather unexpected uniformity insofar as the nitrogenous changes induced by the microbes are concerned.

This picture is one of moderate proteolysis in mediums containing no utilizable carbohydrate, and a suppression of this proteolysis nearly to the vanishing point when utilizable carbohydrates are added.

These changes are not unlike those induced by pure cultures of colon bacilli and Bacillus mesentericus grown under parallel anaerobic conditions, but it must not be inferred that these bacilli are alone concerned: both stained preparations prepared from the several cultures and actual isolations show a considerable variety of forms and types, gram-negative organisms predominating.

Nor is this moderate proteolysis, occurring characteristically in the sugar-free mediums, one free from aromatic products of putrefaction. Indol was present in each plain broth culture after the third day of incubation, and earlier in some. Of theoretical importance, however, is the complete absence of indol in glucose, starch and milk cultures, with the exception of one starch culture (number 2) that had an unusual amount of polypeptid nitrogen.

One of the potentially useful facts gleaned from these observations is the striking effect of diet on the nitrogenous metabolism of these normal adult bacteria: utilizable carbohydrate, as long as it is continuously available, reduces or suppresses aromatic putrefactive products practically to the vanishing point.

Another feature which must be regarded as of tentative significance only, in view of the small number of observations, is the low proteolytic change in plain broth cultures, which contrasts strikingly with the marked proteolysis in those persons whose intestinal flora contains a distinct overgrowth of gas bacilli, Bacillus aerogenes capsulatus.[5]

[5] Study LXXX, this series.

CHEMISTRY OF THE INTESTINAL FLORA OF MAN CONTAINING ABNORMAL NUMBERS OF GAS BACILLI

STUDIES IN BACTERIAL METABOLISM. LXXX

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The organism known as Bacillus welchii, or more commonly the "gas bacillus," because of its ability to induce a violent fermentation in saccharine mediums, is commonly found in the alimentary canals, both of man and of many animals. It is usually considered to be an inhabitant of the lower rather than the upper levels of the intestinal tract. Here it grows with moderate luxuriance, ordinarily a harmless parasite, in spite of the fact that its development is seemingly associated with the formation of butyric acid and a soluble poison.

The organism has also been reported as occurring in the mouth, in apparently normal gallbladders, and in appendices, according to various observers.[1]

The frequence with which the gas bacillus is found in the feces of normal persons varies somewhat in different years,[2] for unknown reasons: that is to say, Bacillus welchii is distinctly more commonly encountered some years than others. This same seasonal irregularity in distribution is not confined to the gas bacillus: it is also met with in the occurrence of Bacillus cloacae, members of the mucosus capsulatus group, and other microbes.

There is no satisfactory explanation at present for this irregularity in frequence, and but few observations on it, yet it is important.

For this reason, it is difficult to distinguish sharply between normal numbers and normal occurrence of the gas bacilli, and their presence in abnormal numbers and abnormal occurrence. Nevertheless, the occurrence of the Welch bacillus does have more than academic interest to the clinician because it is frequently associated in a quantitative relationship

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[1] Simonds, Monograph, Rockefeller Inst. for Med. Res., 1915, No. 5.
[2] Kendall: Southern M. J., 1925, 18, p. 120.

with at least four different syndromes. This quantitative relationship is primarily a definite outgrowth of the organisms, shown by the relatively large number of spores and vegetative forms in the feces; and, in at least some instances, by definite evidence of an extension of the habitat of the microbes upward in the intestinal tract. Also, the virulence of the organisms may be increased.[3] The precise part that the gas bacillus plays, however, is still undetermined.

The four syndromes in which the gas bacilli are unusually numerous are: acute diarrhea, and occasional sporadic diarrhea;[4] pernicious anemia;[5] diabetes;[6] a hitherto undescribed syndrome.[7] With the exception of the diarrheal cases, the part played by the gas bacillus in these very unlike syndromes is problematical. It is much more logical at present to assume that gas bacilli becomes unusually numerous as a result of some change in the intestinal environment, than that the organism is a primary incitant of such unlike conditions as diabetes and pernicious anemia.

This does not preclude the possibility that some intestinal condition may offer the requisite opportunity for one or another strain of gas bacilli to become abundant in these four syndromes mentioned above.

The conditions under which the Welch bacillus may become prominent among the intestinal organisms is not well understod, chemically at least, and therefore a study was made of the intestinal floras of persons having unusually large numbers of gas bacillus spores in their feces. These persons were not acutely ill, but were not entirely well. All of them were of that group benefited by a markedly restricted carbohydrate diet, and a liberal administration of well soured buttermilk.[7]

In all, 17 cases were studied. Three are reported here. Of these, numbers 1 and 3 represent as nearly as possible the extremes of chemical findings encountered in these cases; case 2 shows about an average of these findings.

The methods employed were those described in a previous communication.[8]

DISCUSSION

The most conspicuous feature of the analyses tabulated is the rapid production, and unusually large amount, of free ammonia in the cul-

[3] Simonds, Monograph, Rockefeller Inst. for Med. Res., 1915, No. 5, p. 54.
[4] Ibid., p. 89.
[5] Ibid., p. 72. Moensch, Kahn and Torrey: J. Infect. Dis., 1925, 37, p. 161.
[6] Personal observation: Organisms usually very abundant in diabetics, but with no very obvious connection with the disease.
[7] Kendall: To be published.
[8] See Study LXXVII, this series.

TABLE 1

METABOLISM OF BACTERIA OF FECES OF ADULT. ABNORMAL NUMBERS OF GAS BACILLI

Mediums		Control			1 Day			3 Days			6 Days			9 Days		
		Case 1	Case 2	Case 3	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3
Plain Broth	Nitrogen															
	Total	938.0	917.0	973.0	938.0	917.0	973.0	938.0	917.0	973.0	938.0	917.0	973.0	938.0	917.0	973.0
	Protein	882.0	749.0	749.0	702.9	726.6	681.8	478.8	558.5	547.4	478.8	469.0	446.6	445.2	413.0	357.0
	Nonprotein	56.0	168.0	224.0	235.2	190.4	291.2	459.2	358.4	425.6	459.2	448.0	526.4	492.8	504.0	616.0
	Polypeptid	4.2	114.8	168.0	160.3	115.5	200.9	309.4	201.6	198.8	234.8	207.2	200.2	229.6	232.4	185.8
	Amino	36.4	35.0	35.0	40.6	40.6	49.0	46.8	33.6	36.4	49.4	39.2	102.2	64.4	23.3	105.0
	Ammonia	15.4	18.2	21.0	34.3	34.3	41.3	102.9	123.2	190.4	175.0	201.6	224.0	198.8	248.3	325.5
	Reaction	+0.2	+0.6	+0.7	+1.4	+1.9	+2.2	+1.8	+2.4	+2.6	+2.2	+2.8	+1.6	+1.8	+2.5	+0.6
Glucose Broth	Nitrogen															
	Total	938.0	917.0	973.0	938.0	917.0	973.0	938.0	917.0	973.0	938.0	917.0	973.0	938.0	917.0	973.0
	Protein	882.0	749.0	771.4	747.6	749.0	749.0	803.6	737.8	749.0	803.6	737.8	749.0	770.0	760.2	771.4
	Nonprotein	56.0	168.0	201.6	90.4	168.0	224.0	134.4	179.2	224.0	134.4	179.2	224.0	168.0	156.8	201.6
	Polypeptid	5.6	119.0	147.0	134.4	130.9	166.6	78.4	142.1	164.5	75.6	142.1	165.2	107.8	103.6	138.6
	Amino	35.0	30.8	33.6	38.5	16.8	30.8	37.1	16.1	32.2	37.8	14.7	32.2	36.8	30.8	35.7
	Ammonia	15.4	18.2	21.0	17.5	20.3	26.6	18.9	21.0	27.3	21.0	22.4	26.6	21.0	22.4	27.3
	Reaction	+0.2	+0.9	+1.0	+3.8	+6.2	+6.3	+8.3	+8.9	+10.1	+8.3	+10.1	+10.5	+4.1	+10.2	+9.9
Starch Broth	Nitrogen															
	Total	903.0	903.0	959.0	903.0	903.0	959.0	903.0	903.0	959.0	903.0	903.0	959.0	903.0	903.0	959.0
	Protein	458.2	746.2	768.6	634.2	723.8	723.8	522.2	723.8	768.6	477.4	735.0	701.4	477.4	623.0	723.8
	Nonprotein	44.8	156.8	190.4	268.8	179.2	235.2	380.8	179.2	190.4	425.6	168.0	257.6	425.6	280.0	235.2
	Polypeptid	1.4	110.6	137.2	204.4	123.2	175.0	267.4	92.4	137.2	334.6	83.3	144.2	331.1	158.2	172.9
	Amino	29.8	28.7	33.6	37.8	30.1	29.4	47.6	35.7	33.6	41.3	37.1	36.4	51.1	46.9	31.5
	Ammonia	14.0	17.5	19.6	26.6	25.9	30.8	65.8	51.1	19.6	49.7	47.6	77.0	43.4	74.9	36.8
	Reaction	+0.2	+0.6	+0.8	+2.3	+3.5	+4.2	+2.8	+4.3	+0.8	+7.7	+5.9	+3.6	+5.4	+4.4	+5.8
Milk	Nitrogen															
	Total	499.8	483.0	511.0	499.8	483.0	511.0	499.8	483.0	511.0	499.8	483.0	511.0	499.8	483.0	511.0
	Protein	471.8	438.2	432.6	471.8	438.2	432.6	460.6	438.2	432.6	460.6	427.0	432.6	460.6	427.0	466.2
	Nonprotein	28.0	44.8	78.4	28.0	44.8	78.4	39.2	44.8	78.4	39.2	56.0	78.4	39.2	56.0	44.8
	Polypeptid	5.6	23.8	56.0	2.8	25.2	54.6	14.0	22.4	54.6	15.4	32.2	56.0	14.0	25.2	22.4
	Amino	18.2	13.3	14.0	19.6	11.9	13.3	19.6	13.3	14.0	18.9	14.7	13.3	18.9	21.7	13.3
	Ammonia	4.2	7.7	8.4	5.6	7.7	10.5	5.6	9.1	9.8	4.9	9.1	9.1	6.3	9.1	9.1
	Reaction	+1.5	+1.5	+1.8	+3.6	+5.8	+4.1	+9.5	+12.4	+12.1	+14.1	+14.2	+14.7	+13.2	+14.7	+17.5

Nitrogen recorded in mg. per 100 cc. culture medium; reaction in cc. normal acid or alkali per 100 cc. culture medium, using neutral red as an indicator.

tures in the sugar-free gelatin broth. The smallest amount encountered in any of the samples studied is that of number 1 in which the very considerable increase of 180 mg. has accumulated by the ninth day of incubation. Even on the sixth day, it amounts to 150 mg., indicating a very extensive deamination. There is also a marked cleavage of the protein, shown by the increase in nonprotein nitrogen. Indeed, the nonprotein nitrogen fraction in all of the three samples shows a very considerable increase. This predicates a soluble, very active bacterial proteolytic enzyme, of the order of that produced by typical strains of Bacillus proteus.[9]

The ammonia increase is greatest in number 3. Here it reaches the surprising amount of rather more than 300 mg. per 100 cc. of culture medium; almost ⅓ of the total nitrogen of the medium.

A comparison of these unusually strong manifestations of microbic proteolysis in carbohydrate-free mediums, and the almost complete absence of proteolysis in the corresponding milk mediums in this series, affords both an unequivocal evidence of the sparing action of carbohydrate for protein, and also a suggestion of the conditions under which gas bacilli may thrive in the intestinal environment. Gas bacilli were not found in an active state in any of these milk cultures, inoculated with the entire intestinal flora; nevertheless, the gas bacilli were very abundant in the inocula. The simple expedient of heating the milk to 80 C. for 30 minutes, after inoculation and prior to incubation, resulted in every instance in a rapidly developing "stormy fermentation" from which gas bacilli could be readily demonstrated in nearly pure culture.

A similar instance of the relative unfitness of artificial mediums as compared with the intestinal tract for microbic development was encountered in the somewhat restricted growth of Bacillus bifidus outside the alimentary environment.[8]

Herein seems to be one explanation for the conditions under which Welch bacilli may develop overabundantly in the alimentary canal: an abundance of carbohydrate and a restriction in activity or a reduction in number of normal intestinal lactic acid-forming bacteria that otherwise would tend to restrain the development of the gas bacilli,[10] especially above the large intestine.

[9] Kendall, Cheatham and Hamilton: J. Infect. Dis., 1922, 30, p. 251.

[10] Kendall: Boston M. & S. J., 1913, 169, p. 734. Simonds: Monograph, Rockefeller Inst. for Med. Res., 1915, No. 5, p. 107.

Possibly some weight can be given to this argument by the satisfactory clinical results attained in these cases through reducing the carbohydrate of the diet rigidly, and administering well soured buttermilk in frequent, rather large amounts.

It makes no appreciable difference whether the soured milk contains Bacillus bifidus, acidophilus, bulgaricus, or Streptococcus lacticus in these cases: the prime requisite seems to be preformed lactic acid.

CONCLUSIONS

The outstanding characteristic in the metabolism of the intestinal flora of persons having an overgrowth of gas bacilli in their alimentary tracts is seen in mediums containing no utilizable carbohydrate, as gelatin broth. Here there is considerable and rapid degradation of the protein. This is accompanied by unusually extensive deamination, resulting in the accumulation of much ammonia in the culture.

In carbohydrate-containing mediums, and especially in milk, there is a complete, or nearly complete, suppression of this proteolysis, and of deamination, as well.

It must be emphasized that this characteristic reaction is quantitative, rather than qualitative in its significance, and a much larger series must be available before unassailable conclusions can be drawn.

FATE OF BACT. TYPHOSUM AND OTHER ORGANISMS IN SEGREGATED VEIN AND IN GENERAL CIRCULATION OF NORMAL AND IMMUNIZED RABBITS

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The bacteriologic literature of the latter part of the last century is replete with publications dealing with the disappearance of bacteria in the blood stream and with the mechanism by which the animal body is able to rid itself of infections. The humoral theory supported by Baumgarten, Ziegler, Nutall, von Behring, Ehrlich, and Pfeiffer, and the cellular or phagocytic theory championed by Metchnikoff, Ribbert, Hess, Lister and Bordet, were in active competition for supremacy in this field. The two theories were generally conceded to be mutually exclusive; a point scored for one was considered to be directly against the other.

In later years, immunologists admitted the validity of both points of view, and considered that both factors operate in the destruction of bacteria in the body. The various works of Metchnikoff, Zinsser, Kolmer, and others, give full surveys of the earlier literature and of the interaction of the various ideas put forth. The humoral destruction of bacteria is still vaguely understood, but is recognized as of more or less importance. Phagocytosis, on the other hand, is more tangible, and has grown to be more universally believed in as the most important factor in the cleansing of the blood stream of extraneous materials, whether bacteria, tissue fragments or foreign bodies.

Bull [1] studied the disappearance of typhoid bacilli introduced into the general circulation, by means of heart blood samples and tissue fragments taken at stated intervals after injection into the animal. The impression received from this paper is that agglutination and filtration in the capillary bed of the various organs, followed by phagocytosis, is the chief mechanism for the removal of the bacteria.

During a study of this problem, the thought came to us that it would be instructive if these two factors, humoral and cellular, could be separated, and their individual effects measured. Fixed tissue

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[1] Jour. Exper. Med., 1915, 22, p. 475.

phagocytosis occurs for the most part in the capillary bed of the various organs, as in the spleen, liver, bone marrow, lungs, etc., and little or none in the larger blood vessels, the endothelial lining of which has not been shown to have any phagocytic action. If, therefore, comparable tests could be carried out in the general circulation with the phagocytic mechanism intact, and in a segregated portion of the inferior vena cava where the fixed tissue phagocytic mechanism is presumably the only element lacking, a comparison of the two would give an idea of the relative importance of this form of phagocytosis. The action of the circulating phagocytes would be the same in both cases, unless stasis in the vein induces a change in their activity.

The results which follow represent the more mature experience gained during the series of experiments to be described, omitting a few of the first ones which contained obvious errors that it was possible to overcome. The work is far from complete as furnishing an adequate explanation for the phenomena observed, but it forms a new point of departure for the study of some of them.

Animals Used.—Large rabbits of both sexes were employed for all experiments. None of them, unless otherwise specified, had received injections with any organism of the genus studied, and many had not been used for previous experiments of any kind. All were in good condition and free from visual evidences of disease.

Calculation of the Blood Volume.—The blood volume was estimated to be one fourteenth of the body weight. This figure was used in calculating the dosage of organisms in order to make this comparable in the different tests. The content of the segregated vein was estimated as accurately as possible.

Strains of Organisms Used.—Four strains were employed; 2 were Bacterium typhosum, 1 Staphylococcus aureus, and the fourth a gram-positive anaerobe, Clostridium sporogenes. One strain of Bact. typhosum, "Rawlings," is avirulent for guinea-pigs and rabbits and has been under cultivation for more than 20 years. It agglutinates readily in the presence of immune serum and is subject to marked lysis in the low dilutions (1:2 and 1:10) of some normal and immune serums. It serves as a good antigen and was used for immunizing animals for certain of the experiments.

The virulent strain of Bact. typhosum is known as "55,' and was isolated recently from a local epidemic of typhoid fever. It does not agglutinate readily and has feeble antigenic powers.

Staphylococcus aureus, "Norsenski" strain, has been in stock for some years. The strain of Cl. sporogenes is designated "310-1" in our collection and was isolated by one of us (Dack) from soil.

Cultural and Other Technical Details.—Plain veal infusion agar with a hydroge nion concentration of P_H 7.4-7.6 was used for plating. The agar was tubed 10-12 c c. to a tube, and after melting and inoculating with 1 c c. portions of the various dilutions was thoroughly mixed and poured into plates. The Ringer's solution used in the experiments was prepared according to the Ringer-Locke formula, which may be found in many texts. The P_H was adjusted to

7.6 with HCl. Dilution blanks were made to contain 4.5 c.c. of the diluting fluid and were autoclaved. Distilled water was used in some of the earlier experiments and 0.85% salt solution in all the later ones.

Young cultures (12-24 hours old) were used in all experiments. The organisms, with the exception of Cl. sporogenes, were grown on plain agar slants. The growth was taken up in about 10 c c. of the diluting fluid. A broth culture of Cl. sporogenes constituted the original suspension. Dilutions were made from this suspension in geometrical series of 10 and a plate count made of the viable organisms per c c. of the suspension. One tenth c.c. portions of the 1 : 100 dilution were added to each c c. of the heparinized blood and serum. The same proportionate amounts of the suspension were added to the fluid in the segregated vein and to the general circulation. The 1 : 10 dilution was used for injection into the general circulation and in order to avoid the introduction of the large volume of diluting fluid. All plates were incubated 48 hours before counting.

Injection of the Organisms.—These were made as soon as possible after suspending the organisms to avoid the inevitable decrease in count which occurs when they are allowed to stand for some time in contact with the diluting fluid. Direct injection was made into the segregated vein and into the in vitro mixtures, and by way of the ear vein to reach the general circulation.

Sampling the Mixtures.—As a rule, 0.5 c.c. of the various mixtures was withdrawn at intervals of 5, 30 and 120 minutes and immediately plated in proper dilutions. The samples from the vein and heart were measured in tuberculin syringes graduated to hundredths of a c c. Pipets were used for withdrawing the serum and blood from the tubes. In certain cases, as in withdrawing the blood from the vein, when the total amount was insufficient to permit a 0.5 c c. sample each time, a smaller amount was used, and suitable corrections were made in calculating the dilution. A separate pipet was used in each step of the dilution.

Cultivation of Cl. sporogenes.—Plating was done in the usual manner, after which the Petri dishes, covered with earthenware lids lined with filter paper, were placed inverted in large anaerobic jars. The air was withdrawn with a water pump for 2 hours, reducing the pressure to 0.4 cm. The jars were placed in the incubator for 48 hours, and the colonies counted. Growth and colony differentiation were satisfactory.

Heparin.—The early experiments were performed without the use of heparin, dependence, to avoid clotting of the blood, being placed during the operation on gentle handling of the vein, and, in the case of the drawn blood, on the use of 0.3 c c. of a 10% solution of sodium citrate. In the latter case this was sufficient, but with the former it was never possible to be sure that even small clots had not interfered with the actual reduction in bacteria. Heparin was used in the experiments reported here. The animal under anesthetic was punctured in the heart with a fairly large needle and sufficient blood was withdrawn to supply serum for the test and for agglutination, and 3 c.c. for the test of the whole blood. The latter amount was measured accurately with the syringe used for withdrawing the blood and was placed immediately in a tube containing a sterilized heparin solution in saline somewhat stronger than that advocated by Howell,[2] viz., about 0.5 mg. per 5 c.c. of blood. The tube was immediately shaken and placed in the incubator until inoculated. For use in the vein, a similar solution was injected together with the dose of bacteria, as soon as possible after the segregation of the vein segment. This was sufficient

[2] Am. Jour. Physiol., 1925, 71, p. 553.

to prevent coagulation of the blood within the period of the experiment, thus overcoming the serious objection encountered in the first part of the series.

So many difficulties were met and overcome in the development of the operative technic that it seems wise to include a more detailed description of it than might otherwise be justifiable.

OPERATIVE TECHNIC

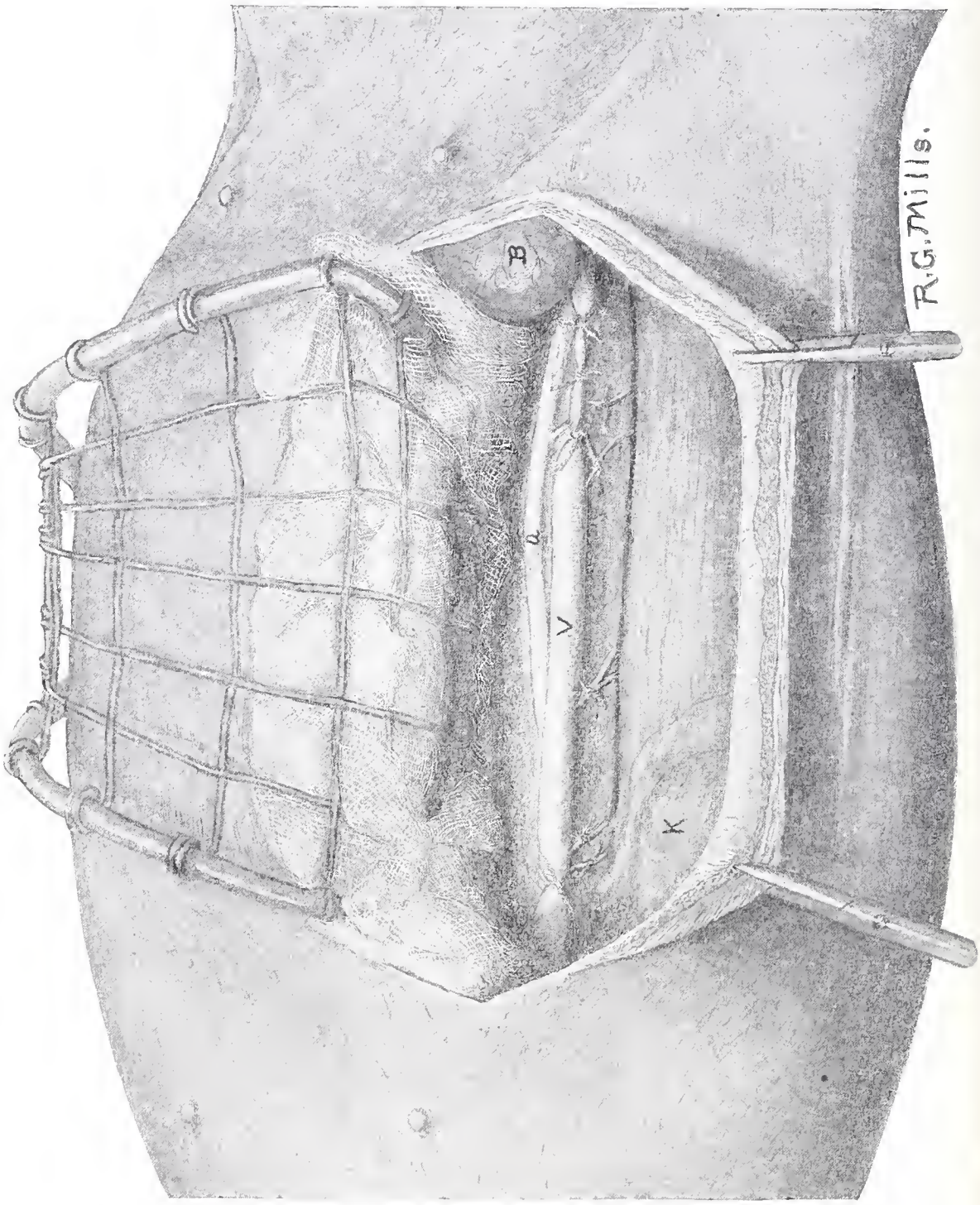
The rabbit was clamped to an animal operating board and the hair clipped from the ventral surface. The area of operation was washed with lysol solution. Under ether anesthesia, an incision was made through the layers of the abdominal wall, about 12 cm. in length, beginning slightly below the costal margin and extending downward toward the groin. This was slightly to the right of the right mammillary line, and severed few blood vessels. The several layers were clamped together with hemostats on both sides of the incision.

The end of a strip of dry gauze, slightly wider than the incision, was tucked first under the peritoneum of the median side of the opening, and then with the remainder the intestines were drawn en masse toward the median line. The visceral mass was kept in place by a retractor attached to a weight, which was allowed to fall over the edge of the table. This retractor was made of a test tube basket, and was 10 cm. wide in the widest portion. This was sufficient to confine all the organs except the bladder, which, in some instances, had to be aspirated with a large needle.

With the aid of a bedside lamp clamped to the edge of the operating board, the field of operation was exposed to intense illumination. The large abdominal vessels were then seen lying in the median line under a little thin fascia. These were then drawn first one way and then another, exposing the thin connective tissue fibers to tension. As these appeared, they were torn apart with fine forceps, carefully avoiding the smaller blood vessels. One by one these were isolated and tied. The number and distribution of these were subject to wide variation, but certain more or less constant features were found. One large tributary vein emptied into the vena cava on the anterior surface and was doubly ligated and incised between the ligatures. The stump was then reflected toward the larger vessel. There were no direct communicating vessels between the aorta and the vena cava, so that with careful dissection the two were completely separated. By drawing the vena cava upward, there were found constantly a number of veins of variable size, passing directly backward between the two masses of muscles anterior to the vertebral column. These were tied in turn and were usually left uncut.

The distention of the receptaculum chyli and numerous branches of the lacteal system occasionally complicated the technic to a slight extent. These vessels overlay the aorta and when distended caused slight inconvenience during the dissection. In such a case these were torn through and completely disregarded, as their walls contained no demonstrable blood supply.

A silk ligature was thrown about the vena cava as close as possible to the juncture with the renal vein, and for the moment left loosely tied. Another ligature was then placed about the vena cava as near the bladder as possible and likewise loosely tied. A convenient ligature carrier was found to be an ordinary probe through the eye of which the thread was passed. The shaft was bent into a wide S shape, with the tip forming a loop to serve as a handle. The flat point was useful in passing between the vein and the artery where the two had not been completely separated. Careful picking at the fascia over the point enabled one finally to force a passage between them without danger of rupturing either. For this purpose the probe was much better than a needle passed eye first in a similar way.



Exposure of abdominal aorta (A) and vena cava (V), showing relations to gallbladder (B) and kidney (K).

The upper ligature was then tied firmly enough to occlude the circulation but not to injure the intima. The vena cava had usually contracted to a marked degree under the influence of the manipulation of its adventitia, but this was overcome by placing one finger over the yet untied lower ligature and with another finger making slight and cautious pressure over the intervening segment of the vein. The walls were thus stretched by moving the finger to the expanded portion and repeating the maneuver. When the vein was sufficiently dilated, the lower ligature was tied, thus completely isolating that segment of the vena cava. Too great distention was not desirable.

Without delay another ligature was placed about the segment a little above the lower thread and tied loosely with one knot. The dose of bacteria mixed with the heparin solution was then introduced into the vein, the operator holding the two ends of the ligature. On completion of the injection, slight traction was put on the ligature, drawing it upward about the needle point. If necessary, the one injecting the bacteria placed his finger on the smaller segment in order to empty it completely. This was necessary only when the second ligature was too far removed from the lowest one. The tying of the last ligature was then completed.

The force of injection was usually sufficient to produce good mixing of the blood with the bacteria, but to make doubly sure of this point, slight massage of the vein was performed.

During the interval between the withdrawal of the samples of blood, the vein was kept covered with moist gauze. Just prior to the withdrawal of the later samples, another ligature was placed about the segment of vein, and the same procedure followed as described above. At the conclusion of the 120 minute period, the entire content of the segment was removed and the amount determined. If the vein was to be removed for histologic purposes or for placing in a test tube with heparinized blood, the intact vein above and below the ligatures was occluded with bull dog clamps, and the intervening part excised.

During the various procedures as little anesthetic as possible was used, just enough to abolish sensation and prevent all movements. A certain degree of shock materially reduced the amount of ether required. At the conclusion of the experiment, the animal was killed with ether.

When the vein was to be washed free from blood with Ringer's solution, the technic was slightly modified. Special effort was made to isolate a segment of the vein as long as possible. The terminal ligatures were tied tightly, with the vein only moderately full of blood. The tip of a fine glass cannula attached to an inverted flask containing sterile Ringer's solution was introduced through the wall of the vein as near one end as possible. At the same time a small nick was made in the wall of the vein near the other end. The original content of blood was soon washed out and the flow allowed to continue for a few minutes until the walls of the vein had been well cleansed. Slight massage of the vessels insured the removal of any blood which might remain in the closed ends of the tied-off tributaries. While the fluid was running, other ligatures were placed in such a position that when tied they would enclose the remaining uninjured segment of vein with its walls fairly well distended with Ringer's solution. From this point the technic was the same as previously described, except that the needle of the syringe containing the dose of bacteria was inserted as for the injection of the organisms, but the contents of the vein were first aspirated into the barrel and measured before reinjecting the mixture. An additional ligature confined the test material within the segregated portion of the vein from which samples were withdrawn as desired. When properly prepared, the fluid in the vein remains absolutely clear, indicating that there is no leak in any of the tributary veins.

Immunization of Animals.—There was no uniformity in the method used for immunizing the animals. In some instances intraperitoneal injections of killed cultures were given on 3 successive days, followed by a rest interval, and then by another series of injections shortly before using the animal. In most cases the titer secured was fairly high. The strain "Rawlings" was used in every case; previous attempts with strain "55" resulted in practically no increase in titer. The homologous strain was not employed in the later experiments because of the marked lysis observed in the low serum dilutions.

Injections of India Ink.—This was a sterilized suspension of ink made by rubbing up stick ink in salt solution. Several c c. of the suspension were injected two or three times during the few days before operation and then again about one-half hour before the experiment began. The organs were darkened in color, and on histologic examination the pigment was found to be present in abundance in the fixed tissue and other phagocytic cells.

SPECIAL TESTS

During the course of the experiments it became necessary to make certain tests as a check on the accuracy and interpretation of the results. Some of these are given below.

1. Agglutination of the organisms by the serum of the animal. This was done in each case and the significance of the findings in any given experiment commented on in that connection. The technic used was that commonly employed. Readings were made a few minutes after incubation, 2 hours later and again the following day, the last interval after placing the racks in the refrigerator.

2. Lysis. In a few instances definite lysis was noted in the agglutination tube with a 1 : 2 dilution, amounting in one case to 85% of the organisms in 2 hours. Usually it was not observable even when checked with hemocytometer counts. This occurred only with the "Rawlings" strain of typhoid.

3. Smears. As a check on the amount of agglutination which might have taken place in the various test solutions, smears were made as a routine in many of the experiments. Some were stained with Wright's stain and some with Manson's. Agglutination had taken place to a certain definite degree but was apparently insufficient to explain even a small part of the marked decrease in the bacterial count.

4. Histologic examination of the vein after conclusion of the experiment. This was made in several instances, and groups of bacteria were found. The number of organisms or location against the wall of the vessel were not sufficient to indicate an undue concentration of bacteria in that locality; nor were the walls unusually sticky to cause the organisms to adhere to them in unusual numbers, thus decreasing their concentration in the fluid contents.

EXPERIMENTAL DATA

A few typical protocols are here reproduced, the remainder appearing in abbreviated form in table 8.

Exper. 1.—Animal, normal; weight, 2280 gm.; organism, "55"; operation, as described.

TABLE 1

EXPERIMENT 1

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	1,564,000	.....	.....
	30	598,000	0.1384	.....
	120	23,550	0.0358	.....
General circulation	5	29,000	.....	.....
	30	50	0.2541	0.000796
	120	20	0.0101	0.000333
Blood	5	1,180,000	.....	.....
	30	620,000	0.0257	.....
	120	15,000	0.0412	.....
Serum	5	990,000	.....	.....
	30	770,000	0.1002	.....
	120	223,400	0.0386	.....

Agglutination 2 hrs., 1:2 dil. ++ at 42 C., none in higher dilutions.

Exper. 3.—Animal, normal; weight, 2075 gm.; organism, "55"; operation, vein segment cut out, slit open and dropped into test tube containing 3 c c. of heparinized blood.

TABLE 2

EXPERIMENT 3

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	1,634,000	.....	.....
	30	805,000	0.03832	.....
	120	11,360	0.0537	.....
General circulation	5	95,000	.....	.....
	30	570	0.2044	0.0000969
	120	160	0.0141	0.0000500
Blood	5	1,695,000	.....	.....
	30	2,159,000	0.0097	.....
	120	25,000	0.0514	.....
Serum	5	3,110,000	.....	.....
	30	1,008,000	0.0450	.....
	120	15,000	0.0480	.....

Agglutination, none in 2 hrs.; complete in 28 hrs. in 1:2 dilution.

Exper. 5.—Animal, normal; weight, 2125 gm.; organism, "55"; operation, routine, plus irrigation of vein before injection of test materials.

TABLE 3

EXPERIMENT 5

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	7,544,000	.....	.....
	30	4,605,000	0.0196	.....
	120	96,965*	0.0444	.....
General circulation	5	16,000	.....	.....
	30	400	0.1473	0.0000972
	120	80	0.0178	0.00011
Blood	5	567,000	.....	.....
	30	500,000	0.00506	.....
	120	125,000	0.01778	.....
Serum	5	490,000	.....	.....
	30	560,000	0.0053	.....
	120	58,000	0.0251	.....
Ringer's solution	5	1,340,000	.....	.....
	30	900,000	0.0159	.....
	120	10,000 (?)	0.0518	.....

Agglutination, none in 24 hours

* A definite decrease in the capacity of the vein was noted, proving conclusively that exosmosis took place through the wall. This had been suspected in a few of the previous experiments, but could not be proved. The amount was not great, but would tend to concentrate the organisms to a slight extent.

Exper. 8.—Animal, immunized to "Rawlings"; weight, 1680 gm.; organism, "55"; operation, routine.

TABLE 4

EXPERIMENT 8

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	1,404,000	.....	.....
	30	470,000	0.0438	.....
	120	2,929*	0.0560	.....
General circulation	5	50,300	.....	.....
	30	760	0.1676	0.000051
	120	30	0.0358	0.000355
Blood	5	1,740,000	.....	.....
	30	1,290,000	0.0187	.....
	120	108,200	0.0274	.....
Serum	5	1,332,000	.....	.....
	30	960,000	0.1050	.....
	120	172,500	0.0190	.....

* Very small clot found at end of experiment, the only instance in which it occurred.

Agglutination, 3 hrs.; 1:400; others negative; next day ++++ up to 1:20 and partial up to 1:1,600.

The count of the original suspension was made merely for temporary use to convey an idea of the relative number of organisms employed. This does not enter into the discussion of the data as it was not possible to use the suspension for all the parts of the experiment simultaneously.

Exper. 10.—Animal, repeated injections with India ink; weight, 2685 mg.; operation, routine.

TABLE 5

EXPERIMENT 10

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	809,900 (?)	.....	.....
	30	887,800	0.00396	.....
	120	936 (?)	0.0758	.....
General circulation	5	162,000	.....	.....
	30	450	0.2342	0.00000884
	120	70	0.0206	0.000133
Blood	5	920,000	.....	.....
	30	840,000	0.0036	.....
	120	209,000	0.0154	.....
Serum	5	1,340,000	.....	.....
	30	770,000	0.02216	.....
	120	155,000	0.0177	.....

Agglutination, none in 2 hours; complete in 24 hours in 1:2 dilution.

Exper. 16.—Animal, normal; weight, 2150 gm.; organism, Staph. aureus; operation, routine.

TABLE 6

EXPERIMENT 16

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	2,024,000	.....	.....
	30	759,900	0.03917	.....
	120	7,820	0.0508	.....
General circulation	5	2,525	.....	.....
	30	360	0.0778	0.0000952
	120	10	0.03968	0.0010800
Blood	5	2,250,000	.....	.....
	30	1,380,000	0.0195	.....
	120	950,000	0.00416	.....
Serum	5	1,180,000	.....	.....
	30	800,000	0.01552	.....
	120	470,000	0.00314	.....

Agglutination, none in 2 hours, complete to 1:200 at 42 C. after 24 hours.

It is known that most bacteria decrease rather rapidly in various menstruums, and hence a comparison with the actual counts in the tests would lead to confusion. The figure representing the supposed decrease in count in the first 5 minutes has been intentionally omitted. In chem-

ical reactions the initial rate of the reaction is commonly so rapid and difficult to measure that it is usually disregarded in computing the curve of the rate of transformation. It is sufficient to determine 3 points in the reaction curve and compute the rapidity from these figures. We propose for the convenience of analysis to evaluate the rate of destruction of bacteria in each case studied by means of single figures. We have chosen to this end to use the constants K_1 and K_2, which can be derived by means of the equations which are commonly used to measure the rate of chemical reactions. The larger the constant, the higher is

Exper. 19.—Animal, normal; weight 2350 gm.; organism, Cl. sporogenes; operation, routine.

TABLE 7

EXPERIMENT 19

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
Vein	5	57,000	.....	.....
	30	20,000	0.0400	.....
	120	125	0.0565	.....
General circulation	5	12,300	.....	.....
	30	100	0.1920	0.000396
	120	20*	0.01782	0.000440
Blood	5	401,000	.....	.....
	30	77,000	0.0660	.....
	120	13,200	0.0195	.....
Serum	5	2,920	.....	.....
	30	840	0.142	.....
	120	310	0.01105	.....

* Marked shock with failing circulation.
Agglutination, 30 minutes ++++ in 1:2 dilution, 4 hours +++ in 1:10, and + in 1:20.

the rate of bacterial destruction. The intervals of examination of 5, 30 and 120 minutes give the observation intervals between them of 25 and 90 minutes. For these the rate of decrease in bacterial count has been computed according to the monomolecular equation or the equation of a first order reaction. In this equation the concentration of the reagents is of no consequence.

The formula for computing the constant, called K_1, of the monomolecular equation is as follows:

$$K_1 = \frac{2.3}{t} (\log. a - \log. b)$$

in which t is the time elapsing between the given observations, a is the count at the beginning of the period and b is the count at its conclusion.

If the rate is constant, the two figures for a given experiment should be approximately equal, allowing for an experimental error. The aver-

age of these two figures is the K_1 cited in table 8. With a complicated system such as the one with which we are dealing, requiring several manipulations for its determination, the chance of a considerable error even in spite of meticulous care is great. In table 8 those figures for K_1 in which there is a close agreement have been marked with a star. It will be noted also that these K_1 values lie entirely in the columns marked vein, blood, and serum in which there are 8, 6 and 6 correlations, respectively. There are none in the column denoting the general circulation.

TABLE 8

RESULT OF INJECTION OF VARIOUS ORGANISMS

No.	Animal	Organism	Vein	Blood	Serum	Average of K_1 for Vein, Blood and Serum	General Circulation	K_2 Values, $\times 0.10^3$ General Circulation
1	Normal	B. typh. "55"	0.0371*	0.0334*	0.0243*	0.0316	0.1321	0.564*
2	Normal	B. typh. "55"	0.024	0.0446*	0.0466	0.384	.....	.....
3	Normal, with isolated vein	B. typh. "55"	0.41*	0.0208	0.465*	0.0361	0.1092	0.0598*
4	Normal, with washed vein	B. typh. "55"	0.015	0.0337*	0.0204	0.0230	0.1097	0.0296*
5	Normal, with washed vein	B. typh. "55"	0.032*	0.0114	0.0099	0.0177	0.0825	0.1036*
6	Normal, with washed vein	B. typh. "55"	—0.0077	0.0131	0.0097	0.0101	0.1138	0.1785*
7	Immunized to "Rawlings"	B. typh. "55"	0.051*	0.0105	0.0218	0.0277	—0.0298	—0.121
8	Immunized to "Rawlings"	B. typh. "55"	0.0499*	0.0230*	0.0620	0.0449	0.1017	0.203
9	Immunized to "Rawlings"	B. typh. "55"	0.0008	0.0113*	0.0170*	0.0097	0.0940	0.285*
10	Normal, injected with India ink	B. typh. "55"	0.0398	0.0095	0.0199*	0.0230	0.1274	0.0709
11	Normal, injected with India ink	B. typh. "55"	0.0648	.....	0.0368*	0.0508	0.0850	0.1036
12	Normal, with isolated vein	"Rawlings"	0.1516	0.1727	0.1566	0.1603	.....	.....
13	Immunized to "Rawlings"	"Rawlings"	0.1195	0.0743*	0.1285	0.1077	0.1326	0.389
14	Normal	Staph. aureus	0.0352	0.0389	0.0118	0.0286	0.0267	0.0239*
15	Normal	Staph. aureus	0.0425*	0.0105	0.0081	0.0203	0.0390	0.0567
16	Normal	Staph. aureus	0.0450*	0.0118	0.0093	0.0220	0.0587	0.587
17	Normal, with isolated vein	Staph. aureus	0.0097	0.0160	0.0281	0.0179	0.0887	0.1365
18	Normal	Cl. sporogenes	.....	0.0086	0.1240	0.0663	.....	.....
19	Normal	Cl. sporogenes	0.0482*	0.0427	0.0765	0.0558	0.1049	0.418*

* Indicates close correlation between K_1 or K_2 values for 25 and 90 minute intervals.

This suggests, therefore, that there is some common factor operating in these three columns which has produced corresponding figures with practically equal frequency. There is only one element which is common to all three, viz., the fluid portion of the blood. The wall of the vein is present only in the first; the cellular elements are present in the first two, and only the fluid portions in the third. It would seem, therefore, that the essential factor in whatever change takes place must reside in the plasma.

In a monomolecular reaction, by definition, there is only one substance undergoing transformation, although Rideal and Taylor [3] have questioned the validity of many of the so-called monomolecular reactions, claiming that a catalyst is also present which is responsible for the change. This may be merely the walls of the test tube, but is nevertheless active in this capacity. The complexity of the blood plasma is too great to allow any theorizing as to the actual changes that take place. Suffice it to say that the three fluids appear to react toward bacteria placed in them in such a manner that the course of the reaction follows that of a reaction of the first order.

The rate of decrease in the bacteria placed in the blood stream is apparently much greater than that operating in the other mediums. The initial count at the end of 5 minutes is very much less than is found in any other mediums, and may even be as low in 5 minutes as the others are after 120 minutes. There is a great and obvious difference. They do not conform to the monomolecular equation in a single instance.

The bimolecular equation represented by the constant K_2 may be expressed as follows:

$$K_2 = \frac{1}{t} \frac{a - b}{a \cdot b}$$

When this was applied to the figures for the general circulation, it was found that there were 8 instances of close correlation. Those which did not closely conform were found to have values which lay intermediate between the two constants and hence could not be expressed by K_3 or by one of the more complicated equations.

It is therefore evident that more forces or factors are in operation in the general circulation than are found in the vein, blood or serum. So far as the analysis has been made, the essential and perhaps the only real difference between the vein and the general circulation is the presence of the agglutination-filtration-phagocytic mechanism emphasized by Bull. If this is a valid assumption, then the difference between the two would be an evaluation of the phagocytic activity of the body; the larger figure, the total bactericidal action of the organism and the values for the vein, blood and serum would represent the bacterial action of the fluid portions of the blood alone.

The data in table 8 have been derived from a series of experiments only a few of the protocols of which are here reproduced. Each horizontal line represents an experiment, the details of which can be reconstructed from the table.

[3] Catalysis in Theory and Practice, 1919.

The effect of the experimental error is somewhat reduced by comparing the average of the two K_1 figures for each curve representing an experiment. These average figures only are given in the several columns in table 8. When these in turn are averaged, the correspondence for a group of experiments, as in the first 6, which deal with normal animals and the strain "55" of typhoid, is even closer. Table 9 illustrates this point.

TABLE 9

AVERAGES OF K_1 VALUES BY GROUPS

Experimental Numbers	Vein	Blood	Serum
1–6	0.0261	0.0261	0.0262
7–9	0.0339	0.1049	0.0336
10–11	0.0523	0.0095*	0.0283
12–13	0.1355	0.1235	0.1425
14–17	0.0331	0.0193	0.1043
18–19	0.0482*	0.0256	0.1002

* One animal only.

Exper. 4.—Animal, normal; weight, 3988 gm.; organism, "55"; operation, routine.

TABLE 10

EXPERIMENT 4

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values
General circulation	5	† 24,719	.....
	30	‡ 0	0.4041
	120	§ 110	—0.0520
Blood	5	390,000	.....
	30	232,000	0.0208*
	120	42,000	0.0189
Serum	5	580,000	.....
	30	384,000	0.0164
	120	517,000	0.0033

* Close correlation.
Agglutination complete in 1:100 in 2 hours.
Distilled water used in dilution blanks; 1% sodium citrate added to blood in vitro.
† Blood from ear vein, ‡ from the heart, § from the aorta.

Evidently the more experiments in a given group, the closer is the correlation. The groups dealing with typhoid bacilli are more nearly uniform than in the case of either of the other organisms used. The rate of destruction is much higher in the experiments when the strain "Rawlings" was used than in any others. This is presumably due to the lysis to which this strain is especially susceptible. Destruction of strain "55" is somewhat more rapid in the fluids from an animal immunized to "Rawlings" than in those from a normal animal. Blocking the phagocytes with India ink has made no appreciable difference in the rate of destruction of the typhoid bacilli.

When an average is struck for each vertical column in table 8, the vein and serum are found to give figures which are nearly the same, and both are slightly above the figure for the blood. The fact that the serum rate of destruction is slightly higher than either of the other two would suggest that no appreciable agglutination had occurred in the vein where it might be expected to take place more readily than elsewhere, thus corroborating the observations mentioned elsewhere that no such phenomenon had been observed.

Exper. 5.—Animal, normal; weight, 2740 gm.; organism, "55"; operation, routine.

TABLE 11

Experiment 5

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
General circulation.....	5	* 9,720	.....	.....
	30	108	0.1800	0.000368
	120	54	0.0077	0.000102
Serum.....	5	247,500	.....	.....
	30	229,750	0.0030	.....
	120	69,670	0.0132	.....

* Heart blood used in this and in all subsequent experiments.
Agglutination ++++ in 1:2 dilution in 2 hours, + in 1:20.

Exper. 7.—Animal, immunized to "Rawlings"; weight, 2100 gm.; organism, "55"; operation, routine.

TABLE 12

Experiment 7

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
General circulation.....	5	11,500	.....	.....
	30	110	0.1809	0.00036
	120	100	0.0010	0.00001
Serum.....	5	5,005,000	.....	.....
	30	2,530,000	0.0272*	.....
	120	75,000	0.0389	.....

* Close correlation.
Agglutination ++++ in 1:10 in 2 hours, ++ in 1:100.
Distilled water used in dilution blanks.

In group 14-17 in which the staphylococcus was used, the rate of destruction appears to be much lower in the blood and serum than in the vein of the same animal, where it is relatively high. This finding hints at the selective action of the fluid tissues of the body in dealing with various bacteria which may invade them. Reference to the details of exper. 16 previously described will show also the great reduction

in organisms in the general circulation at the end of 5 minutes when presumably the same dose of bacteria had been introduced as in the other mediums. The rate of decrease after the 5 minute period is also rapid. The difference between the in vivo experiments on one hand and the in vitro on the other, is quite pronounced. This difference was not seen when the typhoid organisms were used, suggesting strongly that the relative importance of humoral and cellular destruction of bacteria is different for different species of organisms.

Exper. 11.—Animal, normal; injected with India ink; weight, 2425 gm.; organism, "55"; operation, routine.

TABLE 13

EXPERIMENT 11

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
General circulation.....	5	52,650	.....	.....
	30	4,650	0.0962	0.0000078
	120	490	0.0249	0.00002
Serum.....	5	1,395,000	.....	.....
	30	310,500	0.0600	.....
	120	5,050	0.0201	.....

Agglutination ++++ in 1:2 dilution in 1 hour, 1:20 negative.

Exper. 12.—Animal, normal; injected with India ink; weight, 3625 gm.; organism, "55"; operation, routine.

TABLE 14

EXPERIMENT 12

Situation	Interval in Minutes	Bacteria per C c.	K_1 Values	K_2 Values
General circulation.....	5	810,000	.....	.....
	30	6,233	0.1990	0.00000633
	120	790	0.0228	0.0000122
Serum.....	5	1,610,000	.....	.....
	30	934,000	0.0467*	.....
	120	105,000	0.0173	.....

* Close correlation.
No agglutination.

In the preliminary experiments there are some data which, in the light of later developments, are valid. The vein has been excluded from consideration because of the uncertainty existing before the use of heparin to prevent clotting; in a few of the experiments this was definitely proved.

Averaging the constants for the foregoing experiments gives figures closely correlated with those of the main body of experiments

previously described. The K_1 values are: general circulation, 0.1416, average of 5; citrated blood, 0.0198, average of 1; serum, 0.0199, average of 5. Among these experiments there are 3 instances in which occur close correlations of the two figures determining the reaction curve, 1 for the citrated blood and 2 for the serum. The former is possibly of significance as suggesting that citration of the blood does not influence the rate of destruction of bacteria any more than heparinization does. If on further experimentation this should prove to be correct, it might make it unnecessary to replace the use of citrate solution as an anticoagulant in favor of heparin, which, presumably, on theoretical grounds, would be preferable. It might be supposed, if Howell's contention is correct, that heparin is a normal constituent of the blood, that its addition in slightly increased quantities would alter the equilibrium of the blood less than some other substance entirely foreign to it.

SUMMARY

A technic has been developed by which is is possible to study separately the quantitative relations of the humoral and cellular destruction of bacteria in vivo.

Heparin has been shown to prevent the clotting of the blood in vitro and in vivo during the course of certain experiments involving manipulation of the vascular structures.

There was noted a marked reduction in the bacterial counts in the samples taken from the heart blood, segregated vein, heparinized and citrated blood, serum and Ringer's solution used in various experiments.

The rate of reduction in all except the general circulation follows more or less closely the logarithmic or monomolecular type of reaction curve.

The rate of reduction in the general circulation does not conform to the monomolecular equation, but in some instances does conform more or less closely to the bimolecular equation.

The essential difference between the destruction of bacteria in the circulation and that observed in all other mediums examined indicates the presence of at least one additional factor in the former which is absent in the others. This is assumed to be the phagocytic power of fixed tissue cells aided in their action perhaps by agglutination and filtration in the capillary bed of the tissues.

The segregated vein and the general circulation have in common practically every element which could destroy bacteria except that associated with fixed tissue phagocytosis.

The vein and the blood in vitro have in common every factor except the actual presence of the vessel walls. The similarity in the results obtained suggests that this is of no significance in destroying bacteria.

The heparinized and citrated blood in vitro have in common with the serum all the fluid portions of the blood. The last has been deprived, in one form or another, of the cellular elements and of the blood's constituents associated with the clotting mechanism. The similarity in results with these various reagents points to the relative unimportance of the cells of the blood in the active destruction of bacteria as compared with that possessed by the serum alone.

Reduction takes place with greater rapidity in the case of the avirulent strain of Bact. typhosum introduced into the mediums provided by an animal immunized to the homologous strain than when a virulent strain is exposed to the same influences. The same is true when two are placed in mediums taken from the normal animal, but the rate of decrease is not so marked.

The use of India ink to block the phagocytes has little or no effect on the activity of any of the mediums except possibly to lower the rate somewhat in the general circulation.

Destruction of bacteria goes on at essentially the same rate in Ringer's solution in vitro as in a segregated vein containing Ringer's solution left in situ.

The rate of destruction of Staphylococcus aureus in the vein is slightly more rapid between the 5 and 120 minute intervals than in all other mediums, including the general circulation. The reduction in the first 5 minutes, however, was much greater in the latter. This may be associated with the brisk agglutination observed in these cases and which it is supposed may favor their rapid elimination by filtration during the initial portion of the experiment.

The obvious differences between the behavior of Bacterium typhosum and of Staphylococcus aureus may furnish an explanation for the lack of uniformity in the findings of earlier workers, some of whom observed higher and others lower rates of destruction in the blood in vivo than in vitro when working with different species of micro-organisms.

The results attained with Cl. sporogenes are not materially different from those with other organisms. Had they been markedly different, the difficulties encountered in cultivating and counting anaerobic plates would have led us to greater caution in their interpretation.

EFFECT OF CERTAIN SUBSTANCES ON THE PRECIPITIN REACTION

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The work presented in this paper grew out of some incidental observations that the presence of certain carbohydrates somewhat inhibited the formation of a precipitate when immune serum was added to its antigen. It was recognized that this inhibition might be due to the solubility of the precipitate in the reagent, to a partial dissociation of the antigen antibody complex, or to other unknown factors.

A survey of the literature showed that little had been done on substances which inhibit the formation of precipitate in the precipitin reaction, other than Hirsch's [1] work on the inhibitory effect of certain ranges of reaction. Nuttall [2] states that the inhibition of the formation of precipitates in the so-called zone phenomenon is due to the solubility of the precipitate in an excess of antigen. Much has been published, however, on the dissociation of antibodies of various kinds. The work of Kosakai,[3] and Morganroth [4] on the dissociation of hemolysins is well known. Ogata,[5] Gay and Chickering,[6] Chickering,[7] Huntoon [8] and others have offered evidence that agglutinins and precipitins may be dissociated from the formed precipitate.

The object of the following experiments was threefold; first, to study the effect of certain heterologous proteins and carbohydrates on the formation of a precipitate in the precipitin reaction; second, to study the solubility of the formed precipitate in these reagents and in a weak alkali; third, to ascertain whether dissociation of the antigen antibody complex takes place and what relationship this dissociation has to the inhibition of the formation of precipitate by the same reagents.

Since Nuttall [2] suggests that the inhibition of the formation of precipitate is due to a specific solubility of the precipitate in its

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[1] Jour. Infect. Dis., 1923, 32, p. 439.
[2] Blood Immunity and Blood Relationships, 1904, p. 149.
[3] Jour. Immunol., 1918, 3, p. 109.
[4] München. med. Wchnschr., 1903, 50, p. 61.
[5] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1924, 39, p. 270.
[6] Jour. Exper. Med., 1915, 21, p. 115.
[7] Ibid., 22, p. 248.
[8] Jour. Immunol., 1921, 60, p. 117.

antigen, it seemed worth while to try the effect of the presence of large amounts of heterologous proteins on the precipitin test. Antibeef serum was used. The antigen instead of being diluted with salt solution was diluted with the following proteins: normal rabbit serum, normal human serum, 2% aqueous solution of gelatin and 10% aqueous solution of egg white. Serial dilutions made with these proteins were stratified above the undiluted antiserum. Readings were made after 2 hours at 37 C. and again after standing over night in the icebox. Salt solution dilutions of the antigen were used as a control in each test.

No effect was apparent in the amount of precipitate formed nor in the titer of the serum. The prozone effect was not intensified in spite of the large amount of proteins present. These results bear out the suggestion made by Nuttall [2] that the precipitate is soluble only in an excess of its specific antigen.

In studying the effect of heterologous proteins on the formed precipitate from a mixture of antibeef serum and beef serum, the precipitate was washed repeatedly in 0.85% NaCl and then suspended in a minimum quantity of the saline. This suspension was divided volumetrically. One or two volumes of the undiluted human serum, rabbit serum, 2% gelatin or 10% egg white was then added to one volume of the precipitate. The tubes were kept at 37C. for 2 hours. At this time readings were made as to any clearing or visible solution by comparing with a control tube containing precipitate plus saline, it having been previously determined that the precipitate was slightly if at all soluble in this reagent. Each tube was then centrifuged at high speed for 15 minutes and the supernatant fluid removed and used for the following tests: A small portion was stratified above beef serum to test for the possible presence of precipitins. Other portions were diluted with salt solution, usually 1 : 10 to 1 : 100, and stratified above antibeef serum to test for the presence of precipitinogen. In every case a control using normal rabbit serum instead of antibeef serum was used to guard against the presence of nonspecific precipitates. In these tests there was no visible solution of the precipitate, but in the supernatant fluid both precipitins and precipitinogen could be detected. The precipitinogen reacted in dilutions of 1 : 20 to 1 : 50 as a rule, rarely higher. The heterologous protein apparently caused some dissociation, though this was not sufficient to inhibit the formation of the precipitate in an ordinary precipitin test. That this dissociation was not due to the small amount of salt solution present was proved by the fact that

the salt solution extract rarely contained precipitinogen reacting above 1 : 10, and apparently contained no precipitins.

Huntoon has showed that normal human serum causes a slight amount of dissociation of agglutinins and protective antibody from sensitized pneumococci. Our results point to a similar effect on the dissociation of precipitins.

THE EFFECT OF SODIUM CARBONATE

We have not investigated the effect of various concentrations of acid and alkali on the precipitin reaction, since this field has been thoroughly covered by Hirsch.[1] We have, however, tried the effect of weak alkali on the formed precipitate.

If a salt solution suspension of the washed precipitate from any of the antiserums used, antibeef, antiegg or antihuman, is treated with an equal volume of 1% sodium carbonate, almost complete solution of the precipitate takes place. This solution gives a reaction with anti-serum but not with the specific antigen, that is to say, precipitinogen but no precipitin can be demonstrated. However, if the carbonate solution of precipitate is neutralized to P_H 7.4 and is stratified above the antigen, a precipitate forms. It seems, then, that the apparent lack of precipitins is due to interference by the alkalinity of the solution.

Weil,[9] also, found that a sodium carbonate extract of a specific precipitate (native serum plus specific antiserum) contained antigen but no precipitin. Antibodies could be demonstrated, however, by passive sensitization. He assumed the identity of precipitins and the sensitizing antibodies. Huntoon [8] and later Zinsser and Young [10] were unable to demonstrate active agglutinins in sodium carbonate extracts from sensitized pneumococci, although protective antibodies could be demonstrated by mouse inoculation. This is not in agreement with the work of Ogata [5] and Chickering,[7] who state that they were able to demonstrate active agglutinins and precipitins in the sodium carbonate extracts from sensitized bacteria.

It is difficult to compare our results with those of the authors cited, since they do not state whether or not they attempted to neutralize their extracts.

THE EFFECT OF CARBOHYDRATES

The accompanying table summarizes the results of numerous experiments on the effect of carbohydrates on the precipitin reaction and on

[9] Jour. Immunol., 1916, 1, p. 35.
[10] Jour. Exper. Med., 1913, 17, p. 396.

the dissociation of the formed precipitate. We chose these carbohydrates as being representative of the common types used in the laboratory.

Antibeef, antisheep, antiegg-white and antihuman serums were used. In testing for interference with the precipitin reaction, the carbohydrates, to the strength indicated in the table, were dissolved in normal salt solution. Serial dilutions of the antigen were made in these carbohydrate solutions and were stratified above the corresponding antiserum.

EFFECT OF CARBOHYDRATES ON PRECIPITIN REACTION

Carbohydrate	Concentration Used	Degree of Interference	Presence of the Interacting Substance in Extracts of the Washed Precipitate	
			Precipitinogen	Precipitin
Dextrose	2M	Complete	Strong reaction	Strong reaction
Saccharose	2M	Complete	Moderate reaction	No reaction
Lactose	M/2	None	Slight reaction	No reaction
Maltose	M/2	None	Slight reaction	No reaction
Raffinose	M/10	None	Slight reaction	Slight reaction
Mannite	M/2	None	Slight reaction	No reaction
Dulcite	M/5	None	Slight reaction	No reaction
Salicin	M/10	None	Slight reaction	No reaction
Glycerol	M/10	None	Slight reaction	No reaction
Saponin	2%	Slight	Slight reaction	Slight reaction
Saline (control)	.....	None	Slight reaction	No reaction

In testing for dissociation of the formed precipitate, the washed precipitate suspended in a minimum quantity of salt solution was treated with 2 volumes of the carbohydrate of the strength indicated. After standing at 37 C. for 2 hours, the mixture was centrifuged, and the supernatant fluid was used to test for precipitins and precipitinogen. One portion of the supernatant fluid diluted 1 : 10 was stratified against antigen to test for the presence of precipitins. The other portion diluted from 1 : 10 to 1 : 50 was stratified above antiserum to test for the presence of precipitinogens. In the case of dextrose and saccharose, which interfere with the formation of the precipitate, a dilution of 1 : 10 was sufficient to overcome this interference. That this dilution was not too great to demonstrate the presence of precipitin if it were present in any quantity may be seen from the positive results obtained with dextrose.

Since Huntoon [8] showed that distilled water was a more effective dissociating solution for protective antibodies than 0.85% salt solution, it was thought necessary to repeat these experiments using the carbohydrates made up in distilled water. No appreciable differences were observed in the inhibiting effect exerted by the carbohydrate nor in the

amount of dissociation which took place. No essential differences were observed between the different antiserums used. Various concentrations of the carbohydrates used were also tried. Dextrose M/5 gave a slight inhibition only; 4% and 0.4% gave similar results. A concentration of 30-40% was necessary to give inhibition. When the washed precipitate was treated with equal volumes of 40% dextrose, practically complete solution took place. Both precipitin and precipitinogen were readily demonstrated in the diluted supernatant fluid. The effect of the different concentrations of saccharose on the inhibition of precipitate formation was similar to dextrose, but saccharose caused no visible solution of the precipitate and had a much weaker dissociating effect than dextrose. No precipitin was present in the supernatant fluid and only a small amount of precipitinogen.

Different concentrations of the other carbohydrates gave practically no inhibiting effect and caused only slight dissociation of the formed precipitate. If it had been possible to use equally concentrated solutions of the other carbohydrates, this inhibitory effect might have approached that of dextrose and saccharose. That the viscosity of the solution was not the sole cause of the inhibition of the formation of precipitate, however, may be judged from the experiments with gelatin, egg white and glycerol, which did not give any inhibition. Saponin in 2% and 0.2% solution had no apparent effect on the titer of the serum, but the amount of precipitate was less in each dilution than in the salt control.

So far as we are aware, no work has been done on the inhibiting effect of dextrose or saccharose on the precipitin reaction. Ogata,[5] using anticoli agglutinating serum, stated that agglutination took place as well in 10% saccharose as in salt solution. Huntoon[8] found that dextrose 5.6% and saccharose 10% were fairly effective in dissociating agglutinins, bactericidal and protective antibodies from sensitized pneumococci. In our results the inhibiting effect of 20-30% saccharose and 20-30% dextrose and the dissociative effect of the latter are striking. It is hoped that further work may throw some light on the mechanism of the interference of these two sugars on the precipitin reaction. The solubility of the formed precipitate in dextrose suggests that this might be an explanation of the interference with the formation of precipitate. That this explanation cannot apply to the interference by saccharose may be seen from the foregoing results in which saccharose, although interfering with the formation of the precipitate, did not give any solution and only a slight amount of dissociation. In summing up, our

results seem to show that certain carbohydrates may cause inhibition of the precipitin reaction. The proteins used, weak alkali and these carbohydrates cause some dissociation, but there is not a complete parallelism between the amount of inhibition and dissociation. Substances like dextrose and sodium carbonate solution, which cause solution of the precipitate, also cause the greatest amount of dissociation. It might be pointed out that the precipitin reaction offers peculiar difficulties to the study of dissociation, since it is practically impossible under the conditions of the test to measure the quantity of precipitins dissociated.

SUMMARY

The presence of an heterologous protein, such as normal serum, does not interfere with the precipitin reaction nor intensify the prozone, thereby confirming Nuttall's results on the specific solubility of the precipitate in the homologous antigen. Heterologous proteins did, however, cause dissociation of the formed precipitate.

A solution of the precipitate in weak carbonate was shown to contain active precipitinogen but no precipitin.

On neutralization of the carbonate extract both precipitinogen and precipitin were readily demonstrable.

Concentrated solutions of dextrose and saccharose inhibit the formation of precipitate in the precipitin reaction.

Dextrose dissolves the precipitate and apparently causes a true dissociation, since both precipitin and precipitinogen can be demonstrated in the extraction fluid from the washed precipitate.

Saccharose has no dissolving effect and causes only slight dissociation of the formed precipitate.

Other carbohydrates seem to interfere little with the precipitin reaction and act as weak dissociating agents, but they were not used in the same high concentration as the dextrose and saccharose because of lack of solubility.

EXPERIMENTAL METHOD FOR STUDY OF BACTERIAL FLORA OF GASTRO-INTESTINAL TRACT

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The early investigators utilized the feces for the source of material for studying the bacterial flora of the gastro-intestinal tract. On account of the enormous number and varieties of bacteria present, requiring a considerable dilution of the fecal specimen, it was soon recognized that this was only a rough method of determining the bacteria present in the intestinal tract.

Bienstock [1] in 1884 called attention to the fact that the upper part of the small intestine contains relatively few bacteria as compared with the lower part of the small intestine and the whole of the large intestine. The early investigations tended to show that there was a complicated process involved in the maintenance of the bacterial flora of the intestinal tract. It was not a process of addition of ingested bacteria to these already present, but there was some kind of a regulatory mechanism whereby the bacterial flora was kept remarkably constant in composition.[2] This complicated the problem of using the bacterial content of the feces for studying the intestinal bacterial flora.

From this point the investigations branched off in two directions; one problem was the mechanism involved in the reduction of the number of the bacteria in the small intestine; [3] the other problem was concerned with the determination of the intestinal bacterial flora by analysis of the feces.

Fistulous openings at various levels of the intestinal tract have been established, and the material obtained has been studied as to its bacterial content. We have repeated some of this work, but we have discarded all of our results for the reason that we are convinced that such animals are not in a physiologic condition. The higher up in the intestinal tract that one makes a fistulous opening, the quicker the animal becomes dehydrated from loss of fluids, and the more digestion there is around

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[1] Ztschr. f. klin. Med., 1884, 8, p. 1.
[2] Kohlbrugge: Centralbl. f. Bakteriol., I. O., 1901, 29, p. 571; ibid., 30, pp. 10, 70.
[3] Rolly and Liebermeister: Deutsch. Arch. f. klin. Med., 1905, 85, p. 413.

the opening of the fistula. Such dogs have a leukocytosis and an elevation of internal temperature. Then in addition to dehydration there is evidence of an intoxication from a foreign protein source. This would be expected as a result of the digestion or autolysis of the tissue around the fistulous opening. We were unable to avoid the difficulties mentioned, although many methods were tried in an effort to obtain a physiologic preparation. We were convinced that we were dealing with a pathologic reaction, and our results were untrustworthy.

After many attempts, by various procedures, we have finally found that the best results can be obtained by attaching the small intestine to the anterior abdominal wall. Our method is as follows: Under sterile conditions, the abdomen of an anesthetized dog is opened, the desired part of the small intestine is located and attached to the anterior abdominal wall lateral to the line of incision. The peritoneal surface of the intestine is gently scraped with a knife or rubbed vigorously with a piece of gauze for a distance of 5 to 7 cm. opposite to its mesenteric attachment. An incision is then made through the peritoneum, fascia, and muscles to the superficial fascia beneath the skin. The muscles are retracted and the peritoneum and fascia are stitched together over the cut surfaces of the muscles. The segment of the gut is now brought up to the intact skin and held in place by a few stitches through the serosa of the gut and adjacent fascial layer. The incision is now closed in the usual manner. Care must be taken in the choice of the intestinal segments. Traction on the mesentery, twisting, and such changes in position must be avoided. The development of this technic has allowed us to prepare ventral fixations of various portions of the intestinal tract, without producing any evidence of obstruction to the intestine. Such dogs are allowed to rest until the incision is entirely healed. These dogs show every evidence of being in a physiologic condition; weight remains constant or increases, appetite good, temperature normal, and the blood examination shows nothing pathologic. With a needle and syringe one can now obtain a specimen from the ventrally fixed portion of the intestine for bacteriologic study. Such specimens can be obtained as often as desired, and the bacterial content can be studied in its relation to ingested food material. The fate of ingested bacteria can be followed better than under previous experimental conditions. We have some dogs with 5 different levels of the small intestine attached to the ventral wall, and can in this way study the changes that take place in the contents at various levels of the tract. A ventral fixation of the first portion of the duodenum allows one to inject bacteria, chemicals, etc., and then study the subsequent changes that take place at lower levels. In this way the stomach and therefore most of the gastric variables that have heretofore entered into such work are eliminated, and the time factor of the entrance of the material into the small intestine is controlled.

Table 1 gives the average results obtained by the use of our method with 11 dogs. These dogs all have a normal temperature, normal leukocyte count, and showed every evidence of being in a physiologic condition.

This work has been controlled by experiments. We have obtained from the corresponding levels of 31 dogs, under ether anesthesia, specimens for bacteriologic and chemical study. The results check with those obtained by the ventral fixation method. We have every evidence to believe that the aspirated material can be considered as presenting the contents of a normal intestinal tract.

TABLE 1

BACTERIAL FLORA AND HYDROGEN ION CONCENTRATION OF THE SMALL INTESTINE (POST-DIGESTIVE) of HEALTHY DOGS

Portion of Small Intestine	Bacterial Flora	P_H
Duodenum	Few gram-positive cocci	5.2 to 6.0
Jejunum, upper half	Few gram-positive cocci	5.5 to 6.5
Jejunum, lower half	Gram-positive cocci, few gram-positive and gram-negative bacteria	6.0 to 7.0
Ileum	Very rich bacterial flora	6.8 to 8.0

SUMMARY

By fixing certain segments of the small intestine to the anterior abdominal wall, specimens can be obtained for both physiologic and pathologic study.

BACTERIAL FLORA AND HYDROGEN ION CONCENTRATION OF DUODENUM

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The contents of the duodenum and the greater part of the jejunum are almost free of bacteria during the post-digestive periods. The bacteria present usually consist of members of the gram-positive cocci group, staphylococci, and enterococci, and at times isolated gram-negative bacilli are encountered. Bacteria ingested with the food are destroyed before reaching the ileum, which contains a very rich bacterial flora.

Rolly and Liebermeister [1] in 1905 reviewed the literature and carefully repeated the work of previous investigators as well as making many new observations in relation to this question of the "auto-sterilization" of the upper part of the intestinal tract. They found that pancreatic juice, trypsin, bile, and scrapings of the intestinal mucosa did not exert a detectable bactericidal effect; in fact, they found that these substances could be utilized as growth promoting factors in proper concentration. Bacteria introduced into the upper end of the intestinal tract or into closed healthy intestinal loops were killed. Eiskamp and Park [2] have recently substantiated this observation. Rolly and Liebermeister concluded that the healthy intact intestinal mucosa played the greatest rôle in sterilizing the contents of the upper portion of the small intestine.

Nothing of fundamental importance was contributed to this problem until 1921, when Gander and Van der Ries [3] reported some observations made on the intestinal flora by using an ingenious apparatus. These workers used a hollow cartridge that could be opened and closed by an electric magnet. The position of the cartridge was determined by the distance from the mouth and by fluoroscopic examination. In this way, they were able to introduce bacteria or chemicals at any level of the intestinal tract, and also to remove specimens for bacterial examination from any chosen segment. They showed that bacteria introduced into the duodenum were destroyed before reaching the ileum in healthy

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[1] Deutsch. Arch. f. klin. Med., 1905, 85, p. 413.
[2] J. Infect. Dis., 1921, 28, p. 67.
[3] Deutsch. Arch. f. klin. Med., 1921, 137, p. 348.

persons, and that this took place irrespective of diet. They also showed that this mechanism was as potent during active as during post-digestive periods.

Van der Ries [4] found a constant bacterial floral in the empty post-absorptive small intestine. There were 4 groups normally present: a Streptococcus lacticus, Bacillus lacticus, Bacillus lactis aerogenes, and Bacillus coli communis. These workers mention an "auto-disinfection" of the upper part of the small intestine and not a "sterilization" process, as it is never sterile, but has its obligate bacterial flora that is as constant as is the particular flora of any other part of the intestinal tract.

There have been many contributions from observers, using duodenal aspirations, on the bacterial flora of the duodenum. It is difficult to exclude salivary and gastric contaminations. The passage of the tube through the pyloric opening, its apposition with the gastric mucosa, introduces possible abnormal secretory and motor disturbances. The mucosal motor mechanism [5] as well as the outer muscular coats are stimulated by the presence of a foreign body in contact with the duodenal mucosa. Boardman [6] has recently shown that bacteriologic findings based on duodenal aspirations must be checked carefully with the flora present in the oral and gastric regions. We have recently described an experimental method of obtaining bacteria from various levels of the gastro-intestinal tract of animals.[7]

McClendon and his co-workers [8] have reviewed the literature up to 1920 on the subject of hydrogen ion concentration of the contents of the small intestine. These workers found the contents of the duodenum of healthy men to range from P_H 4.1 to 6.5, the period of observation lasting for 5 days. Many reports are found of the H-ion concentration of the duodenum since the general use of the duodenal tube. Some observers find a variation from P_H 4 to P_H 8. We have felt that the former represented a recent outpouring of gastric contents: P_H 8 represents a recent secretion from the pancreas not mixed with gastric contents, but most of the results are found to be between P_H 5 and 6.5.

We wish to mention briefly some of the factors that influence the H-ion concentration of the contents of the duodenum:

(1) Gastric contents. We mean by this the total and free acidity of the contents. This includes the digesting as well as the fasting stomach.

[4] Ztschr. f. d. ges. exper. Med., 1923, 34, p. 385.
[5] King and Arnold: Am. J. Physiol., 1922, 61, p. 80.
[6] Am. J. Med. Sc., 1924, 167, p. 847.
[7] Arnold, L.: J. Infect. Dis., 1926, 38, p. 246.
[8] J. Am. Med. Assn., 1920, 75, p. 1639.

(2) Secretions from the pancreas. These are alkaline in reaction and probably continuous with increased amount of secretion when digestible material is present in the duodenum.[9]

(3) Secretions from the liver. These secretions are alkaline, but are not so well buffered as are the pancreatic secretions.[10]

(4) Secretions from the mucosa of the duodenum. These are reported to be alkaline in reaction, but their composition is as yet unknown.

The mixture of the materials from these 4 sources represents the contents of the duodenum. The H-ion concentration is dependent on many factors, but we have found that the H-ion concentration of the duodenal contents of the dog was constant in its concentration despite the theoretical possibilities of variations due to the factors mentioned above.

TABLE 1

BACTERIAL FLORA AND H-ION CONCENTRATION OF THE SMALL INTESTINE OF A FEVER DOG

Portion of Intestine	P_H	Flora
Duodenum	6.9	Heavy growth
Upper jejunum	7.2	of mixed
Lower jejunum	7.4	bacterial
Ileum	7.5	flora in all

EXPERIMENTS

The ligation of the pancreatic ducts does not lead to a change in the bacterial flora of the duodenum. This was done on 2 dogs with ventral fixation of the duodenum. They were observed for a period of 4 months. Specimens were taken from the duodenum each week. Both the flora and H-ion concentration were the same as in control animals. Carlson has shown that elevation of body temperature of the dog is accompanied by a depression of gastric secretion. We caused an elevation in temperature by injecting staphylococcus vaccine. We found that the bacterial flora in the duodenum was the same as that in the ileum and cecum and the H-ion concentration changed to neutral or slightly alkaline (P_H 6.8 to 7.4). When the temperature and leukocyte count returned to the normal level and the dog reacted normally, the duodenal flora and H-ion concentration returned to normal. Table 1 shows the results of acute experiments, as well as material obtained from ventrally fixed segments of the small intestine.

[9] Luckhardt, Stangl and Koch: Am. J. Physiol., 1923, 63, p. 397; McClure, Montague and Campbell: Arch. Int. Med., 1924, 33, p. 535; McClure, Montague and Mortimer: Boston Med. & Surg. J., 1924, 190, p. 357; Gyotoku, M.: H. a. d. med. Fakul. d. Kaisere Univ. z. Tokyo, 1923, 30, p. 409.

[10] Okada: J. Physiol., 1915-16, 50, p. 14.

Alkaline phosphate buffer solution (P_H 8.9) was injected directly into the duodenum of a normal dog. This was injected at the rate of 10 cc. per minute after warming to 37 C. Dog 6 in table 2 illustrates the changes that take place in the bacterial flora, temperature, peripheral leukocytes, as well as the changes in the types of leukocytes. The bacterial flora was determined by putting one drop of the aspirated contents of the duodenum on the surface of an agar plate and smearing with the a sterile bent glass spreader. The blood was obtained from the ear of the dog. The bacterial flora changes from the type usually found in the duodenum to that usually encountered in the ileum or cecum. The leukocytes increase in the peripheral circulation. Arneth's index shows a great variation in the distribution of the leukocytes. The temperature of this dog fluctuated within the normal range during the experiment. Dog 7 (table 2) had a stitch infection and a conjunctivitis

TABLE 2

CHANGES IN BACTERIAL FLORA OF DUODENUM AND PERIPHERAL LEUKOCYTES AFTER ALKALINIZATION OF THE DUODENUM

Bacterial Flora	Time	Temperature	White Blood Cells	Differential Count					
				Polymorphonuclears	Lymphocytes	Mononuclears	Transitionals	Eosinophils	Arneth's Index
Dog 6, before injection, 4 colonies enterococci	9:50 a.m.	101.2	9,200	64	17	11	4	4	36
	10:30	101.2	18,600	85	12	1	1	1	36.0
	11:00	101.4	18,800	87	9	2	2	..	26.0
After experiment, 150-200 colonies mixed flora	11:30	101.0	19,000	94	3	1	2	..	34.5
	12:00	100.8	21,200	87	12	..	1	..	52.5
Dog 7, before injection, 150-200 colonies mixed flora	8:20 a.m.	103.4	35,500	90	7	..	1	..	45
	9:00	102.4	29,600	87	9	1	1	2	42
	9:30	102.8	43,200	91	8	..	..	..	48
After experiment, 200-250 colonies mixed flora	10:00	102.6	36,200	88	9	1	2	1	53.5
	10:30	102.8	41,300	92	5	3	1	..	33.5

50 cc. of a phosphate buffered solution P_H 8.9 (37 C.) injected into duodenum. Dog 6 injected at 10 a. m. Dog 7 at 8:30 a. m.

in one eye. His temperature was elevated and there was a leukocytosis in the peripheral circulation. The bacterial flora in the duodenum at the beginning of the experiment was the same as that found in the large intestine. The injection of the alkaline buffered solution did not influence the duodenal flora. There is a shifting of Arneth's index during the course of the experiment.

Table 3 shows the same experiment with these 2 dogs a few days later. In this instance the same amount of acid buffer solution (P_H 2.6) was injected into the duodenum. There was no change in the peripheral distribution of the leukocytes, and Arneth's index was constant in dog 6. The bacterial flora did not change as a result of the acid buffer injection. and the types of cocci found were those associated with the obligate duodenal bacterial flora. There were no gram-negative bacilli in the duodenum. Dog 7 did not show a change in flora after the acid buffer injection. There is an interesting shifting of Arneth's index 30 minutes after the injection.

TABLE 3

CHANGES IN BACTERIAL FLORA OF DUODENUM AND PERIPHERAL LEUKOCYTES AFTER ACIDIFICATION OF THE DUODENUM

Bacterial Flora	Time	Temperature	White Blood Cells	Differential Count					
				Polymorphonuclears	Lymphocytes	Mononuclears	Transitionals	Eosinophils	Arneth's Index
Dog 6, before injection, sterile	8:45	101.8	16,400	86	12	..	2	..	36
	9:30	101.8	16,500	89	11	1	..	1	40
	10:00	101.8	16,200	85	13	..	2	..	39
After experiment, 50 colonies enterococci	10:30	101.6	16,000	83	15	..	2	..	30
	11:00	101.6	15,600	81	16	1	2	1	30
Dog 7, before injection. 125 colonies mixed flora	9:15	102.0	40,000	86	10	1	..	3	51
	10:00*	101.2	34,700	90	6	2	1	1	66.5
	10:30	101.2	41,500	90	5	4	1	..	33
After experiment, 150 colonies mixed flora	11:00	101.4	36,800	88	8	2	2	..	42
	11:30	101.6	52,400	91	7	..	1	..	34

* 1 normoblast.
50 cc. of solution (P_H 2.6) (37 C.) injected into duodenum. Dog 6 at 9:00; dog 7 at 9:30.

Dog 22: Ventral fixation of duodenum. Temperature, 102.1 F.; white blood cells, 23,000; 40 cc. of P_H 3 buffered solution injected into duodenum. After 30 minutes, white blood cells 22,800; after one hour, 7,800; after 1½ hours, 20,800; after 2 hours, 27,200. Bacterial flora in the duodenum was normal and remained so during the experiment.

Dog 23: Ventral fixation of duodenum. Temperature 101.6 F., white blood cells, 13,200. 40 cc. of a P_H 5 buffered solution injected into the duodenum. After 30 minutes, white blood cells, 9,000; after 1 hour, 12,600; after 1½ hours, 9,800; after 2 hours, 7,600. The duodenal flora remained normal throughout the time of the experiment.

Dog 9: Ventral fixation of the first part of the duodenum and the middle of the upper third of the jejunum. Operated on Sept. 21, 1924; duodenal puncture, Oct. 8, no growth. Jejunal puncture, mixed flora, 150 colories, Staphylococcus

aureus predominating. 50 cc. of P_H 6 buffered solution injected into duodenum. Two hours later there was no change in the flora of the 2 levels.

Duodenal puncture performed October 15. No growth. Jejunal puncture, 100 colonies, staphylococci predominating; 50 cc. of P_H 6 buffered solution injected into the duodenum. 30 minutes later the flora of the 2 levels had not changed.

On October 21, abscess developed at point of fixation of jejunal segment.

Dog 10: Same preparation as dog 9. Stitch infection. Temperature 103.8 F.; duodenum, 200 colonies mixed flora. Jejunum, 300 colonies mixed flora; 40 cc. of P_H 7.6 buffered solution injected into duodenum. Two hours later the flora of the 2 segments had not changed.

Dog 11: Same preparation as dog 9. Operated on Sept. 27, 1924. On September 30, the dog developed "snuffles"; nasal and bilateral conjunctival exudation. Temperature, 103.6 F.; 50 cc. of a P_H 2.7 buffered solution was injected into the duodenum. The duodenal flora (200 colonies) was of a mixed type, B. coli predominating before the injection. The colonies were fewer (75), but B. coli predominated 2 hours after the injection. The jejunum showed the same flora as the duodenum and changed in the same way after the acid injection. The following day the temperature was 104.2 F. Dog anesthetized and flora and H-ion concentration of small intestine determined before killing animal. Duodenum, upper, middle, and lower thirds of jejunum and ileum were tied off into loops. The bacterial flora was the same from the pylorus to the ileocecal valve. The H-ion concentration varied from P_H 6.9 in duodenum, P_H 7 in upper and middle thirds, and P_H 7.2 in lower third of jejunum, P_H 7.4 in ileum.

Dog. 13: Same preparation as dog 9. Twelve days after operation, dog normal. Temperature, 102 F.; duodenum, no growth; jejunum, 20 colonies, enterococci, 50 cc. of P_H 9 buffered solution injected. After one hour, 100 colonies from same amount of duodenal contents as before, B. coli predominating. Jejunum showed a heavier growth, almost pure culture of coli. H-ion concentration of fluid obtained from lower segment was P_H 8.2.

We have protocols from 27 dogs, covering over 150 experiments. The protocols mentioned above are illustrations of certain typical reactions. Dogs 22 and 23 illustrate the leukopenia one sometimes encounters in the peripheral circulation. We find this after acid injections in the most instances, but leukocytosis has been observed occasionally. Leukocytosis is the rule after alkaline injections, but leukopenia has been found in 2 dogs. The brief protocols of dogs 9 to 13 illustrate the effect of acid and alkaline buffers injected into the duodenum. Acid solutions have little effect on the bacterial flora. If the H-ion concentration is beyond the buffer range (P_H 3), there is a vigorous peristalsis. This probably accounts for the slight decrease in the number of bacteria following injections. The bacterial floral soon return to the previous level. If there is a rich flora, the alkaline solutions do not exert a detectable effect on the number of bacteria present; if the flora is normal, the alkaline solutions cause a marked increase in the number and the appearance of large intestinal types of bacteria in the duodenum.

When the duodenal contents are alkaline or neutral in reaction, produced as a result of gastric secretory disturbances or the injection into the duodenum of alkaline buffer solutions, we find a bacterial flora in this region that is abnormal in that it is the flora usually observed in the ileum and cecum. In these levels of the intestinal tract, the alkaline or neutral reaction is normal.

The injection of an acid buffer solution into the duodenum does not correct this disturbed mechanism. Such solutions stimulate peristalsis and are soon passed out of the small intestine. We are now working on some methods that may allow a gradual slow injection of such acid solutions, but our results so far are negative as to the readjustment of the flora. After alkaline solutions are injected, the appearance of the B. coli-aerogenes type of flora takes place within 1 or 2 hours, and lasts for from 3 to 9 hours. The addition of acid solutions after the alkali does not prevent the cecal type of flora from developing or appearing in the upper part of the small intestine, but does cause the readjustment to take place sooner. This observation was not constant in every experiment.

SUMMARY

When the normal reaction of the contents of the duodenum and upper jejunum is changed from a slightly acid (P_H 5 to 6) to a neutral or alkaline reaction (P_H 7 to 8), there is a change in the bacterial flora of this region.

When the contents of the duodenum and upper jejunum are neutral or alkaline in reaction, the bacterial flora resemble that found in the ileum and cecum.

The normal duodenal flora is not changed by tying off the pancreatic ducts.

The maintenance of the normal H-ion concentration of the contents of the duodenum and upper jejunum is dependent to a great extent on normal gastric secretory function.

HOG CHOLERA STUDIES *

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The following experiments relate to the transmission of hog cholera and the longevity of the virus in blood and the body excretions. The blood and excretions used for exposing and inoculating the different lots of experimental pigs were obtained from pigs that were killed from 5 to 9 days after the intramuscular injection of a highly virulent virus. All of the pigs were obtained from farms in a section of the state in which outbreaks of the disease had not occurred for years and vaccination against cholera is not practiced. This furnished as uniform a virus as could be produced. The susceptibility to hog cholera of all the pigs giving negative results or remaining well in all of the following experiments was proved by the injection of fresh cholera blood. All contracted cholera. Body temperatures were not taken, as admittance to pens or yards was not permitted. The diagnosis of hog cholera or visible symptoms of hog cholera were confirmed by necropsies.

PEN EXPOSURE EXPERIMENTS

In order to approximate natural conditions, duplicate yards (30 x 30 feet) were constructed with new lumber on bluegrass pasture that had never been used for hogs. The yards were separated by spaces of 4, 8 and 16 feet in width. three trials were made with yards that were 4 feet apart; two trials were made with yards 8 feet apart and one trial was made with yards 16 feet apart. Three or more healthy pigs were placed in each of the 12 yards. The pigs in 6 of the yards were inoculated with cholera blood, while the pigs in the opposite yards were left for exposure. Each yard was provided with a sun shade and metal drinking fountain.

The results of this experiment are shown in table 1.

TABLE 1

Results of First Experiment

No. of Pigs Inoculated	Duration of Symptoms, Days	Distance Between Yards, Feet	Number of Pigs Exposed	Duration of Exposure, Days	Results of Exposure
9	5 to 32	4	3	32	1st cholera symptom 30 days
4	5 to 21	4	4	21	1st cholera symptom 16 days
4	3 to 19	4	4	19	1st cholera symptom 19 days
4	5 to 21	8	4	21	1st cholera symptom 11 days
4	4 to 22	8	4	22	1st cholera symptom 17 days
13	3 to 47	16	4	50	Remained well 50 days

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During the early fall season healthy pigs were exposed to inoculated pigs in a new hog house, 12 x 8 feet. Two board partitions 3 feet apart, were built through the center of this house, making two pens. The partitions were made by placing inch boards on edge to a height of 3 feet, from which point wire netting was continued to the ceiling, so that pigs would not get into the space between the pens. Three litters of 6 pigs each were placed in this house at different times, 3 pigs being inoculated with cholera blood and 3 pigs left untreated for exposure. After each exposure the house was cleaned and disinfected. As a result of these exposures, the exposed pigs developed cholera symptoms in 15, 20 and 23 days after the inoculation of the pigs in the opposite pen.

As cholera was transmitted consistently across a small space regardless of board partitions, one of the partitions was covered over the entire surface with a good grade of cheese cloth and the experiment repeated. Three inoculated pigs showed symptoms from the 3rd to the 16th day; 4 additional pigs were inoculated and showed symptoms from the 16th to the 30th day of the experiment. Three exposed pigs in the opposite pen remained well for 47 days. The experiment was repeated; 3 inoculated pigs showed symptoms from the 3rd to the 31st day. Three exposed pigs remained well for 33 days.

The cheese cloth curtain was removed and the experiment repeated. The inoculated pigs showed symptoms from the 4th to the 16th day. The exposed pigs developed cholera on the 11th day of the experiment.

The cheese cloth curtain was replaced; cholera was maintained in one pen from the 4th to the 12th day. The exposed pigs in the opposite pen remained well for 18 days, 6 days after all pigs that had cholera were dead.

Three healthy pigs (litter mates) were placed in each of 2 hog houses 48 hours after the removal of sick and dead cholera hogs. One house was neither cleaned nor disinfected; the pigs in this house developed symptoms on the 5th day and died later of cholera. The other house was cleaned and disinfected immediately after the removal of the hogs that had cholera; the pigs in this house remained well for 21 days.

Ten hogs that had cholera were kept in a grass yard until 5 had died, when the remaining hogs were removed and the yard left vacant for 18 days. Three pigs were then kept in this yard for 30 days; they remained well. Eight pigs were exposed in 2 other yards 3 and 4 days after the removal of sick and dead cholera hogs; these pigs also remained well for 30 days. During November 4 pigs were exposed in another yard 2 days after the removal of cholera hogs; one pig developed cholera on the 8th day; the other 3 pigs developed symptoms 6 days later. The experiment was repeated in July. Four pigs each were placed in two yards two days after the removal of cholera hogs; both groups of pigs remained well for 21 days. Four pigs were then placed in another yard 24 hours after the removal of cholera hogs. During this time the yard received direct sunshine for 8 hours. One pig developed cholera on the 9th day; the other pigs developed symptoms from 6 to 8 days later.

LONGEVITY OF VIRUS

An attempt was made to determine the longevity of virus in blood, feces, urine, eye secretions, and skin scrapings. A number of healthy pigs were fed sterile soil, to which blood, urine and feces of hogs that had cholera had been added. Necropsies of these hogs showed well marked, acute hemorrhagic lesions of hog cholera. The blood, urine and feces were obtained 7, 8 and 9 days after the injection of cholera blood and mixed with the soil to saturation.

The mixtures were protected from direct sunlight; exposed to the air at room temperature from 1 to 6 days, crumbled and fed. Samples of the blood of the hogs furnishing the material for each of these 12 feeding experiments proved to be virulent when injected into other pigs. The blood-soil mixtures were virulent when fed 4 and 5 days after collection. The feces-soil mixtures proved to be virulent only once in 11 feedings, and that on the 4th day after collection. In this case it is probable that the feces became contaminated with blood when removed from the ceca. The urine-soil mixtures were not virulent in 12 feedings made 1, 2, 3, 4, 5, 6 and 18 days after collection.

Fresh urine and feces were collected from hogs with cholera, 5 and 7 days after the injection of cholera blood; these collections were mixed with equal parts of sterile soil and fed to 6 lots of pigs. One lot of pigs fed fresh feces-soil mixture developed cholera on the 5th day; the feces had been collected on the 7th day of cholera. The pen control pigs developed cholera on the 13th day. All of the other pigs, including the pen control pigs, remained well for 18 days.

Fresh urine and feces, collected from hogs with cholera 7 days after the injection of cholera blood, were fed directly to pigs. The pigs that received feces developed cholera on the 6th day; the pen control pigs developed cholera on the 12th day. The pigs that received the urine and the pen control pigs remained well for 18 days.

Fresh urine and feces collected from hogs with cholera 11 days after the injection of cholera blood were also fed directly to pigs. The pigs that received the fresh feces remained well for 21 days. The pigs that received the fresh urine developed cholera on the 6th day; the pen control pigs developed cholera on the 12th day. The experiment was repeated with fresh urine and feces obtained from another group of hogs with cholera 11 days after the injection of cholera blood. The pigs that received the fresh feces remained well for 21 days. The pigs that received the fresh urine developed cholera on the 4th day; the pen control pigs developed cholera on the 8th day.

Fresh blood was collected from hogs with cholera 7 days after the injection of cholera blood. These hogs showed well marked acute hemorrhagic lesions of cholera. The blood proved to be virulent on injection into other pigs, and 60 c c. were sprinkled on each bale of new wheat straw that was used for bedding in 23 concrete pens. The straw was placed in the pens 1, 2, 3, 4, 5, 6, 7 and 8 days before the pigs were kept in them. The pens were bedded deeply, as the attendant did not enter or clean the pens during the first week of the experiment. Four pigs were placed in each pen; water was supplied in metal troughs with a nose; the ration consisted of shelled corn thrown onto the bedding. The results of these exposures are shown in table 2. The experiment was discontinued on the 14th day.

The results of these exposures were irregular and probably depended on chance ingestion of the virus. All subsequent exposures were made by direct feeding or intramuscular injections.

Cholera blood desiccated for 18 and 20 days in vacuum over sulphuric acid at 0 C. produced cholera on injection, but did not produce cholera after 26 days of desiccation. Since blood is not subject to such conditions in natural outbreaks, fresh cholera blood was dried in thin layers on sterile plane glass plates at room temperature. The plates were placed on muslin over wire racks, covered with bell jars and protected from direct sunlight, simulating natural farm conditions. Blood collected from several hogs with cholera that were killed 6 days after injection was exposed to the air for 1, 5, 6, 7, 8 and 9 days. It was then suspended in sterile normal salt solution to the original volumes by trituration in sterile mortars. The 6 suspensions of dried blood were fed

and injected into 12 lots of pigs; each lot contained 3 treated pigs and 2 untreated or pen control pigs. Three pigs were fed 4 c c. and 3 pigs received intramuscular injections with 2 c c. of each suspension. Blood exposed to the air for 24 hours produced cholera in 5 days when fed and injected; the pen control pigs developed cholera on the 9th day. Blood that was dried for 5 days was not virulent when fed, but when injected symptoms were produced in 5 days; the pen control pigs developed cholera on the 10th day. Blood that was dried 6 days produced symptoms on the 7th day after feeding, and the pen control pigs developed cholera on the 12th day; the pigs that received injections showed symptoms on the 5th day and the pen control pigs developed cholera on the 8th day. Blood that was dried 7 days produced symptoms in 5 days after feeding and injection; both lots of pen control pigs developed cholera on the 11th day. Blood that was dried 8 and 9 days did not prove to be virulent when fed or injected; the pigs remained well for 21 days.

TABLE 2

RESULTS OF SECOND EXPERIMENT

No. Pen	No. Days Between Contamination and Exposure	Results of Exposure
1	1	Hog cholera symptoms on the 5th day
2	1	Hog cholera symptoms on the 5th day
3	1	Hog cholera symptoms on the 6th day
4	2	Hog cholera symptoms on the 5th day
5	2	Hog cholera symptoms on the 6th day
6	2	Remained well
7	3	Hog cholera symptoms on the 5th day
8	3	Hog cholera symptoms on the 10th day
9	3	Remained well
10	4	Hog cholera symptoms on the 5th day
11	4	Remained well
12	4	Remained well
13	4	Remained well
14	5	Hog cholera symptoms on the 5th day
15	5	Remained well
16	5	Remained well
17	5	Remained well
18	6	Remained well
19	6	Remained well
20	6	Remained well
21	6	Remained well
22	7	Remained well
23	8	Remained well

A comparative study was then made of the virulence of blood, eye secretions, and skin scrapings collected from hogs with cholera 5 days after inoculation. The eye secretions consisted of the accumulations in the canthi and on the lids, and swabs made from wiping the margins and medial surfaces of the lids. The skin scrapings were obtained by carefully shaving the skin of the cheeks and jowls, areas that are usually injured in fighting. These collections were dried for 8 days as in the preceding experiment, and then suspended in 45 c c. of sterile normal salt solution by trituration in sterile mortars. In all of the following experiments 3 pigs were fed 10 c c. each with dose syringes, and into 3 other pigs 5 c c. each of the same suspension were injected intramuscularly. Each lot of pigs was kept in an individual house; two additional pigs were used as pen controls in each house. The blood used in every experiment proved to be virulent when fresh. The results of drying for 8 days are as follows: The pigs fed and receiving injections with dried blood developed symptoms of cholera on the 3rd day; both lots of pen control pigs developed cholera on the

7th day. Pigs that were fed the eye secretions showed symptoms on the 3rd day, and the pen control pigs on the 10th day; pigs that received injections with the eye secretions showed symptoms on the 3rd day, and the pen control pigs on the 8th day. Pigs that were fed and injected with the skin scrapings showed symptoms on the 3rd day, and the pen control pigs on the 7th day.

Blood, eye secretions and skin scrapings were collected from hogs with cholera 6 days after inoculation, and dried for 9 days. All of the pigs that were fed the 3 suspensions, and the pen control pigs, remained well for 14 days. The pigs into which the dried blood was injected showed symptoms on the 4th day, and the pen control pigs on the 8th day. The pigs into which the eye secretions were injected showed symptoms on the 4th day and the pen control pigs on the 11th day. The pigs into which the skin scrapings were injected and the pen control pigs remained well for 14 days. Eye secretions collected from another group of cholera hogs and dried 9 days proved to be virulent when fed and injected into pigs. The pigs that were fed showed symptoms on the 8th day and the pen control pigs on the 13th day; the pigs that received injections showed symptoms on the 3rd day and the pen control pigs on the 9th day.

Blood obtained from another group of hogs with cholera 6 days after inoculation and dried for 10 days produced cholera in 4 days when injected, but did not produce cholera when fed.

Blood and eye secretions were collected from hogs with cholera 11 days after inoculation and dried for 11 days. Pigs fed the blood showed symptoms on the 9th day and the pen control pigs on the 14th day; pigs into which the blood was injected showed symptoms on the 5th day and the pen control pigs on the 9th day. The pigs that were fed the eye secretions and the pen control pigs remained well for 14 days; the pigs into which the eye secretions were injected showed symptoms on the 4th day, and the pen control pigs on the 8th day.

Blood and eye secretions were collected from hogs with cholera 7 days after inoculation, and dried 12 days. Pigs that were fed the blood and the pen control pigs remained well for 14 days; the pigs into which the blood was injected showed symptoms on the 6th day, and the pen control pigs on the 10th day. The pigs that were fed the eye secretions showed symptoms on the 6th day, and the pen control pigs on the 11th day; the pigs into which the eye secretions were injected showed symptoms on the 5th day, and the pen control pigs on the 10th day.

Blood and eye secretions were collected from hogs with cholera 7 days after inoculation, and dried for 13 days. The pigs that were fed the blood and the pen control pigs remained well for 14 days; the pigs into which the blood was injected showed symptoms on the 6th day, and the pen control pigs on the 10th day. The pigs that were fed the eye secretions showed symptoms on the 7th day, and the pen control pigs on the 11th day; the pigs into which the eye secretions were injected showed symptoms on the 6th day, and the pen control pigs on the 10th day.

Blood and eye secretions were collected from hogs with cholera 7 days after inoculation, and dried for 15 days. The pigs into which the blood was injected showed symptoms on the 6th day and the pen control pigs on the 12th day. All of the other pigs remained well for 14 days.

The experiment was repeated. Blood and eye secretions were collected from 9 hogs with cholera 7 days after inoculation, and dried for 15 days. One pig into which the eye secretions were injected developed cholera on the 6th day; one pig into which the blood was injected remained well. Blood and eye secretions were collected from another group of cholera hogs 7 days after

inoculation. The eye secretions were dried for 14 days; the pigs fed and receiving injections with these secretions remained well. The blood was dried for 15 days; the pigs fed and receiving injections with this blood also remained well. All of the pigs proved to be susceptible.

SUMMARY

The results of exposures to hog cholera, made outdoors during the summer seasons, show that hog cholera is easily transmitted over short distances. However, when the distance approximates that of the average width of farm lanes, the disease is not easily transmitted. Similar occurrences have been observed in field outbreaks.

The results of exposures made indoors during the winter seasons show that hog cholera can be transmitted consistently across small spaces regardless of ordinary pen partitions and without direct contact; also that such transmissions can be prevented by placing a cheese cloth curtain between the pens.

Hog cholera virus was viable in closed pens 2 days after the removal of sick and dead cholera hogs. The virus also remained alive in grass yards for 2 days during the month of November, and for 1 day during the hot weather of summer. However, in both cases only 1 of 4 pigs developed the infection, indicating that pigs may escape picking up infection in large yards.

Mixtures of sterile soil with blood, feces, and urine of hogs with cholera were dried at room temperature for 6 days. The blood and soil mixtures remained virulent for 5 days. With possibly one exception, the feces-soil and the urine-soil mixtures were not virulent 24 hours after being removed from the bodies of hogs with cholera.

Fresh urine and feces were virulent at different periods after inoculation. There are indications that the feces become virulent before the urine, and that the virulence of the urine persists longer than that of the feces.

Pigs developed hog cholera when exposed to dry straw bedding on which blood had been sprinkled 5 days previously.

Blood dried from 1 to 15 days at room temperature proved to be virulent when fed or injected.

Eye secretions dried under the same conditions were virulent from 1 to 13 days. In one instance eye secretions proved to be virulent after 15 days of drying.

Skin scrapings dried 8 days were also virulent when fed and injected, but did not produce cholera after 9 days of drying.

A COMPARATIVE STUDY OF STRAINS OF CLOSTRIDIUM CHAUVOEI OBTAINED IN THE UNITED STATES AND ABROAD

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The cultural and pathogenic characters of Clostridium chauvoei as described by various authors differ in many important respects. Two types of the Rauschbrand bacillus are recognized in Germany, the Foth [1] type and the Kitt [2] type. In the description of both these types the original authors differentiated them from the chain-forming rods, Foth being especially insistent on this point. Zeiszler [3] considered that spontaneous blackleg, traumatic blackleg, and parturient blackleg of cattle, La Mancha, Reinderpest and Walfisch blackleg were all due to the Rauschbrand bacillus, while the diseases of sheep, swine, and wild pigs were due to Ghon-Sachs bacillus. Recently Zeiszler [4] and also Kojima [5] described the Foth and Kitt types and studied many of their characters. The type they studied as Rauschbrand Kitt has been identified with Vibrion septique by Meiszner,[6] who suggests the term Pararauschbrand for this organism. Knorr,[7] Gaiger,[8] Leclainche and Vallée [9] also identify the Kitt type or Pararauschbrand with the Vibrion septique. Meiszner [6] considers the Foth or true Rauschbrand bacillus as the cause of blackleg of cattle, Pararauschbrand being the cause of parturient blackleg of cattle and of the similar diseases of other animals. Both organisms have been found associated in certain cases of all forms of blackleg. Heller [10] isolated both C. chauvoei and C. oedematis in material obtained from three cases of blackleg in cattle.

Franklin and Haslam,[11] and Haslam and Franklin [12] isolated an organism from a blackleg spore vaccine (Blacklegoids) which they

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[1] Ztschr. f. Infectionskrankh. der Haustier., 1910, 6, p. 201; 8, p. 117.
[2] Centralbl. f. Bakteriol., I. O., 1887, 1, pp. 684, 690, 716, 723, 741.
[3] Berl. klin. Wchnschr., 1919, 56, pp. 107, 488; Centralbl. f. Bacteriol., Ref. 1, 1919, 70, p. 197.
[4] Ztschr. f. Infect., parasit., Krank. u. Hyg. d. Haustier., 1920, 21, p. 1.
[5] Ztschr. f. Immunitätsf. u. exper. Therap., 1923, 37, p. 170.
[6] Centralbl. f. Bacteriol., I. O., 1922, 89, p. 123.
[7] Zentralbl. f. d. ges. Hyg. u. i. Grnzbte., 1923, 4, pp. 81, 161.
[8] J. Comp. Path. & Bacteriol., 1924, 37, p. 163.
[9] Compt. rend. Acad. d. sc., 1913, 156, p. 123.
[10] J. Infect. Dis., 1920, 27, p. 385.
[11] Ibid., 1916, 19, p. 408.
[12] Ibid., 1920, 26, p. 424.

described as pseudoblackleg. This organism had many of the characters of C. chauvoei, but blackened ferrous sulphate agar and did not protect animals against typical blackleg cultures. In the same year Robertson [13] reported on the isolation of B. oedematis-maligni, Koch, from Blacklegoids.

Weinberg and Séguin [14] found that B. oedematis maligni was usually either C. sporogenes or C. sporogenes containing Vibrion septique. These authors isolated C. sporogenes and C. bifermentans from a strain of B. oedematis maligni obtained from the Lister Institute by Miss Robertson.

Recently Leclainche and Vallée,[14a] in discussing their studies with blackleg material, suggest that this disease should be considered as gas gangrene of cattle and that it is due to several associated anaerobes, as is the case in human gas gangrene. These authors find that spontaneous blackleg is due to C. chauvoei but that parturient and traumatic blackleg and the blackleg diseases of other animals are due to C. septicus. Intermediate types have been isolated. In many cases these strains do not protect animals against either of the typical strains. C. septicus isolated from cattle is shown to be nonpathogenic for this species, but it is pathogenic for horses. C. chauvoei, on the other hand, is pathogenic for cattle but not for horses. In conclusion they suggest that the best vaccine for blackleg would be one produced according to their dysgenic method (Leclainche and Vallée [9]) and containing all the types found in this disease.

Immunization experiments and field results during the past 10 years (Haslam,[15] Scott [16]) have shown that blackleg biologic products, blackleg filtrate produced by filtration of the fluid portion of pure cultures of C. chauvoei and blackleg aggressin, the filtered muscle juices of cattle injected with pure cultures of C. chauvoei, have reduced losses from blackleg to less than 1 in 10,000, whereas before vaccination was practiced losses in the affected districts of the southwest were from 10 to 25% of all young stock.

Products produced from atypical strains, a toxic product and products having a marked putrefactive odor have not proved their efficiency. Franklin and Haslam [11] and Haslam and Franklin [12] show that vaccines produced with their pseudoblackleg strain do not protect calves or guinea-pigs against true blackleg.

[13] J. Path. & Bacteriol., 1916, 20, p. 327.
[14] La Gangrene Gazeuse, Monograph de l'Inst. Pasteur, 1918.
[14a] Compt. rend. Acad. d. Sc., 1924, 178, p. 2024.
[15] J. Immunol., 1920, 5, p. 539.
[16] Kansas Agric. Exp. Sta., Technical Bull., 1923, 10, p. 24.

Graüb,[17] in discussing the results obtained from the use of Graüb and Zschokke nontoxic filtrates, finds that losses are reduced to less than 1% and that these losses are due to C. oedematis in many cases.

In this investigation strains of C. chauvoei isolated in Kansas since 1910 and used in the production of efficient blackleg biologics are used as a standard for the comparison of strains obtained from California, Scotland, England, Germany, and Switzerland.

A French strain obtained from a vial of liquid blackleg vaccine from the Pasteur Laboratories was compared with the Kansas strains in 1918-1921, and found to be identical with these strains.

Recently Zanolli and Sordelli [18] identified the causative agent of the blackleg-like disease of Argentina, La Mancha, with the blackleg strains of Zeiszler. Schlingman [19] has also identified the organisms of La Mancha from Argentina, Manqueira disease of Brazil and a strain from New South Wales with blackleg strains of Parke, Davis & Co. These strains are identical with the Kansas strains. Sigwart,[20] in describing the occurrence of blackleg in South Africa, states that the causative agent of this disease was identified by Zeller of Berlin as the Foth type Rauschbrand.

STRAINS INVESTIGATED

The blackleg strains used were Kansas strains 1, 3, 27 and 28. Strains from other sources used were strain Hall, obtained in 1923 from Prof. I. C. Hall;[21] strain Berg, obtained from the Berg Biological Laboratories; strain Reddish,[22] obtained from Dr. G. F. Reddish of the University of Maryland; strain Lister, obtained from the Lister Institute, London; strain Gaiger, obtained from Professor Gaiger of the Veterinary College, Glasgow.

Strains Kitt and Foth were obtained from the laboratory of Professor Wassermann. Strains 2,381 and 845 were obtained from Doctor Graüb of the University of Bern. The latter strain in our hands has proved to be a difficult organism to cultivate.

These strains of C. chauvoei were compared with 7 strains of C. oedematis. Types 1, 2, and 3 of C. oedematis were obtained from the

[17] Schweiz. Arch. f. Tierheilk., 1924, 66, p. 33.
[18] Compt. rend. Soc. de biol., 1924, 91, p. 1035.
[19] Am. Vet. Med. Assn., 62 Annual Meeting, 1925. In press.
[20] Berl. tierarztl. Wchnschr., 1921, 37, p. 305; Centralbl. f Bacteriol., Ref. 1, 1921, 72, p. 374.
[21] J. Infect. Dis., 1922, 30, p. 445.
[22] J. Bacteriol., 1924, 9, p. 13.

Lister Institute. Strain sheep was obtained from Dr. Beach of Wisconsin, who recently described this organism in connection with a disease in sheep.[23] Strain 037737 was isolated in 1919 from material obtained from a calf. Strain K.D.C. was isolated from a dairy cow that died of a disease clinically diagnosed as blackleg. Strain Braxy was obtained from Professor Gaiger and was isolated from a case of Braxy. Two strains of C. novyi were also used, Hall and Gaiger.

TABLE 1

PATHOGENICITY OF STRAINS USED. RESULTS RECORDED BY M L D

	Rats	Guinea-Pigs	Rabbits	Calves	Swine
C. chauvoei 1	N.P. 0.1	0.05-0.6	N.P. 1	5–15	.....
C. chauvoei 3	N.P. 0.1	0.1-0.4	.....	5–15	.....
C. chauvoei 23	.....	0.1-0.3	.....	5–15	.....
C. chauvoei 27	.....	0.4	.....	15	.....
C. chauvoei 28	.....	0.1-0.8	N.P. 0.5	5–15	.....
C. oedematis 1	.....	0.025	N.P. 1	.....	.....
C. oedematis 2	.....	0.005	0.5	.....	.....
C. oedematis 3	.....	0.05	.....	.....	.....
C. oedematis K. D. C	.....	0.05	.....	N.P. 20	.....
C. oedematis 037737	N.P.	0.05	N.P. 1	N.P.*	.....
C. oedematis sheep	.....	0.01	N.P. 1	.....	.....
C. oedematis Braxy	.....	0.05	.....	.....	.....
C. novyi Hall	.....	0.3-0.6	.....	N.P. 20	.....
C. novyi Gaiger	.....	0.3-0.6	.....	.....	.....
C. chauvoei Hall	.....	0.2-0.6	N.P. 1	15	N.P. 15
C. chauvoei Gaiger	.....	0.2	.....	20	.....
C. chauvoei Berg	.....	0.5	.....	.....	.....
C. chauvoei Lister	.....	0.3-0.6	.....	.....	.....
C. chauvoei Reddish	.....	0.3-0.8	.....	N.P. 20†	.....
Rauschbrand Kitt	.....	0.1	.....	.....	.....
Rauschbrand Foth	.....	0.3	.....	.....	.....
Rauschbrand 2381	.....	0.3	.....	.....	.....
Rauschbrand 845	.....	N.P.	.....	.....	.....

N.P. indicates nonpathogenic in dosage indicated. Figures indicate M L D in mils. or in certain cases the only dose used.

* Report concerning pathogenicity of strain 037737 obtained from Dr. F. Breed of Jensen Salsbery Laboratories.

† M L D for guinea-pigs at this time 0.8 mil.

PATHOGENICITY

The pathogenicity of anaerobes for various animals and the lesions produced in guinea-pigs have been used as a basis for the differentiation of the strains.[4] In the study of the strains used, the lesions produced in guinea-pigs were very similar for all types studied. The lesions produced by C. chauvoei were less marked except in the muscles; those produced by C. novyi strains showed extensive black subcutaneous edema; those produced by C. oedematis showed a more pinkish coloration of the muscles. None of these types of lesion could be considered of diagnostic importance. Pathogenicity of various strains for laboratory animals depends on the strains used. Some indication of diagnostic

[23] J. Am. Vet. Med. Assn., 1925, 20, p. 632.

importance may be obtained from pathogenicity tests on rabbits. Some C. oedematis strains are pathogenic for these animals but none of the blackleg strains tested show any pathogenicity for this species. Pathogenicity for cattle is of great importance, since no strains except typical C. chauvoei have been shown to be pathogenic for these animals.

The pathogenicity of the strains studied is given in table 1, the minimum lethal dose for guinea-pigs being indicated. In tests on other animals the dose used to kill, or which failed to kill, is indicated. This table shows that C. chauvoei is nonpathogenic for rats in 0.1 c c. doses, or for rabbits in doses of 1.0 c c. All strains were pathogenic for cattle in doses of from 5 to 10 c c. Strain 1 was used to kill a large number of calves in the production of blackleg agressin between 1916 and 1921, at which time its virulence decreased rapidly. C. chauvoei cultures killed guinea-pigs in doses of 0.05 to 0.8 c c. Cultures of C. oedematis were somewhat more pathogenic for guinea-pigs, and usually killed these animals more rapidly; the M L D was from 0.005 to 0.1 c c. In the one case tested on calves, 20 c c. of culture produced no lesions in a calf susceptible to blackleg. C. novyi cultures were pathogenic for guinea-pigs in doses of from 0.3 to 0.6 c c. Strain Hall was nonpathogenic for calves. Throughout the work in guinea-pigs wide variations in the susceptibility of individual animals was observed, and conclusions from comparative pathogenicity in this species must be made with reservations.

TOXICITY

It has been shown that filtrates of brain-liver cultures of C. chauvoei, tissue filtrates from spontaneous cases, and from animals killed by the injection of cultures of C. chauvoei were nontoxic for guinea-pigs when administered in doses up to 25 c c. subcutaneously and 2 c c. intracardially.[24] Haslam and Lumb[25] also demonstrated that toxicity had no relationship to the efficiency of blackleg filtrates.

MORPHOLOGY

Slides made from the fluid portion of 24 hour brain-liver medium cultures of C. chauvoei strains showed a small number of single gram-positive rods with rounded ends. Impressions made from the peritoneal surface of guinea-pigs inoculated with these cultures showed a similar picture. Oval subterminal spores were rarely found and pairs, usually

[24] J. Bacteriol., 1925, 10, p. 265.
[25] J. Infect. Dis., 1919, 24, p. 362.

end to end, were unusual. C. oedematis strains in culture slides show gram-positive rods with rounded ends, pairs were found in some strains. The organisms were considerably more plentiful than in C. chauvoei preparations. In peritoneal impressions nearly all strains showed a number of long chains formed of filamentous rods, from 3 to 4 times the length of single organisms. These organisms had square cut ends. Single rods and pairs were also found. Some strains (type 2 and in certain cases type 1) showed no chains but a number of paired organisms, some of which formed obtuse angles one with another. Culture preparations from C. novyi strains showed single and occasional paired organisms; peritoneal preparations, single and paired rods.

Strain Hall showed single rods in both culture and peritoneal slides. Strain Berg showed a few paired rods in peritoneal impressions. Strain Reddish showed pairs in both culture and peritoneal preparations. Strain Gaiger showed only single rods. Strain Foth showed some paired rods in peritoneal impressions. Strain Kitt showed long chains of filamentous rods in peritoneal impressions. Strain 2381 showed a few pairs in peritoneal impressions. Strain 845 showed single gram-positive rods with rounded ends in culture preparations.

COLONY FORMATION

Deep colonies in 2% liver agar were studied; C. chauvoei strains produced pin point or very small lenticular colonies and only small amount of gas; C. oedematis strains produced lenticular or wooly colonies and produced considerable amounts of gas; C. novyi cultures produced lenticular colonies having a tuft of filaments projecting from one surface, usually the lower, and some of these colonies appeared as double or in a few cases triple disks. There was considerable variation in the form of the colonies in the individual strains under different conditions.

BIOCHEMICAL REACTIONS

Coagulated Serum.—None of the strains studied liquefied this medium. Inoculations were made into the depths of solidified serum, and these were incubated for 7 days in the phosphorus anaerobic jar.[26]

Gelatine.—This medium was prepared according to a method communicated by Prof. I. C. Hall, beef infusion gelatine containing 10 to 15% Difco gelatine, 1% peptone, 0.5% salt, 0.1 or 0.2% dextrose, is prepared, adjusted to P_H 7.2 and tubed without filtration. To maintain

[26] J. Bacteriol., 1922, 7, p. 277.

anaerobic conditions one-half to three-fourths inch of petrolatum is added to each tube, the medium is then sterilized at 10 pounds for 45 minutes. Inoculation is made by means of a capillary pipet through the petrolatum previously liquefied by holding the tube in the flame for a few seconds. When tested in this manner C. chauvoei strains liquefied the gelatine and produced a slight amount of gas; C. oedematis strains liquefied the medium and produced a moderate amount of gas; and C. novyi strains liquefied the medium, produced some gas, and blackened the white precipitate found in the bottom of the tubes. The reactions of the other strains is indicated in table 3, all producing slight gas and liquefaction, except strain 845, which produced no gas, and strain Reddish, which produced a moderate blackening of the precipitate.

CARBOHYDRATE FERMENTATIONS

For use in determining the carbohydrate fermentations of these strains 3 liquid mediums were used: 1% peptone infusion broth made sugar-free by the growth of B. saccharolyticus, Enlow's synthetic medium prepared without the addition of agar, and Enlow's medium made with 1% instead of 0.5% peptone. Brom-cresol purple was added in each case as an indicator. These mediums were stratified with ½ to ¾ inch petrolatum. One mil. of a 5% solution of the sugars was added to each tube containing about 10 mil. medium; the tubes were then steamed for 10 minutes to liquefy the petrolatum, thus allowing the sugar solution to mix with the medium (table 2).

Goss et al.[27] showed that typical C. chauvoei strains would not grow in 2% glucose infusion agar. This reaction has been tested on 40 strains of C. chauvoei at the Kansas State Agricultural College, none of which grow in this medium. All the strains studied were inoculated into freshly boiled and rapidly cooled 2% glucose agar. The results of this test are shown in table 3.

The fermentation reactions presented in table 2 show that C. chauvoei strains are rather delicately saccharolytic. In several instances none of the strains fermented any of the carbohydrates. Under more favorable conditions, which were not exactly determined, glucose, lactose, and sucrose were fermented. When sugar-free infusion or Enlow's medium containing 1% peptone were used, glycerol was occasionally fermented.

[27] J. Infect. Dis., 1921, 19, p. 408.

TABLE 2

SUMMARY OF CARBOHYDRATE FERMENTATIONS

	Glucose		Glycerol		Lactose		Mannose		Salicin		Sucrose		Control	
	Sugar-Free	En-low	Sugar-Free	En-low	Sugar-Free	En-low	Sugar-Free	En-low	Sugar-Free	En-low	Sugar-Free	En-low	Sugar-Free	En-low
Cl. chauvoei..	A G	A G	0	0	A G	A G	0	0	0	0	A G	A G	0	0
Cl. oedematis	A G	A G	a g	0	A G	A G	a g	0	A G	A G	a g	0	a g	0
Cl. novyi.....	A G	A G	A G	0	0	0	0	0	0	0	A G	0	0	0
Cl. chauvoei Hall.....	A G	A G	0	0	A G	0	0	0	0	0	A G	A G	0	0
Cl. chauvoei Gaiger.....	A G	A G	0	0	0	0	0	0	0	0	0	0	0	0
Cl. chauvoei Berg.....	A G	a g	0	0	a	a	a	0	a	a	A	0	0	0
Cl. chauvoei Lister.....	A g	A g	0	0	A G	a g	0	0	0	0	A G	a	0	0
Cl. chauvoei Reddish.....	A G	A G	A G	0	a g	0	0	0	0	0	a g	0	0	0
Rauschbrand Kitt.....	A G	A G	a g	0	A G	A G	a g	0	A G	A G	a g	0	a g	0
Rauschbrand Foth.....	A g	A G	a	0	a g	A G	0	0	0	0	A G	A G	0	0
Rauschbrand 2381.....	A G	A G	0	0	A G	A G	0	0	0	0	A g	a g	0	0

A G, large amounts of acid or gas, respectively, a g, small amounts of acid or gas.

TABLE 3

SUMMARY OF THE DIFFERENTIAL CHARACTERISTICS OF STRAINS STUDIED

	Gelatine	Milk	2% Glucose Agar	Coagulated Serum	Deep Agar Colonies	Impressions from G-P. Liver
Cl. chauvoei.....	Gas; liquefaction	Acid; gas	0	0	Pin point	Single
Cl. oedematis.....	Gas; liquefaction	Acid; gas	Growth	0	Spherical; woolly	Filamentous chains
Cl. novyi.....	Gas; black; liquefaction	Acid; gas	Heavy growth	0	Lenticular	Single; paired
Cl. chauvoei Hall....	Gas; liquefaction	Acid; gas	0	0	Pin point	Single
Cl. chauvoei Gaiger..	Gas; liquefaction	Acid; gas	0	0	Pin point	Single
Cl. chauvoei Berg....	Gas; liquefaction	Acid; gas	Growth	0	Pin point	Single; paired
Cl. chauvoei Lister..	Gas; liquefaction	Acid; gas	Growth	0	Pin point; lenticular	Single
Cl. chauvoei Reddish	Gas; black; liquefaction	Acid; gas	Heavy growth	0	Pin point; lenticular	Single; paired
Rauschbrand Kitt...	Gas; liquefaction	Acid; gas	Growth	0	Spherical; woolly	Filamentous chain
Rauschbrand Foth..	Gas; liquefaction	Acid; gas	0	0	Lenticular; woolly	Single; paired
Rauschbrand 2381....	Gas; liquefaction	Acid; gas	0	0	Pin point	Single

The C. oedematis strains fermented glucose, lactose, and inulin under all conditions. In the case of mediums containing 1% peptone, glycerol, mannose, and sucrose were also fermented, as were also the sugar-free check tubes.

C. novyi in 1% peptone mediums fermented glucose strongly, sucrose moderately and glycerol in some cases. Glucose was the only sugar fermented in the 0.5% peptone Enlow's medium.

The fermentation reactions of the other strains as shown in table 2 show that strains Hall, Gaiger, and 2381 are typical of C. chauvoei. Strain Berg, Lister, and Foth have atypical reactions in 2% dextrose

TABLE 4

Agglutination Reactions

		Rabbit Antiserums									
	Horse Serum 46	Cl. chauvoei 1	Cl. chauvoei Hall	Cl. oedematis 1	Cl. oedematis 2	Cl. oedematis 3	Cl. oedematis sheep	Cl. oedematis 037737	Cl. chauvoei Lister	Cl. chauvoei Reddish	Cl. chauvoei Berg
Cl. chauvoei 1.....	240	**480**	240	0	40	0	...	...	40	0	0
Cl. chauvoei Hall....	240	480	**960**	0	40	0	...	...	80	40	40
Cl. oedematis 1.....	40	40	40	**640**	80	80	...	...	240	40	0
Cl. oedematis 2.....	40	40	80	40	**960**	40	...	...	80	40	80
Cl. oedematis 3.....	0	0	80	40	80	**640**	...	...	80	40	240
Cl. oedematis sheep..	160	...	80	480	320	160	**160**	80	...	160	...
Cl. oedematis 037737.	80	...	80	80	320	640	0	**640**	...	80	...
Cl. chauvoei Lister..	240	80	240	80	240	40	...	...	**240**	240	40
Cl. chauvoei Reddish	80	40	0	80	80	80	...	...	240	**480**	240
Cl. chauvoei Berg....	80	80	40	40	80	80	...	...	40	240	**240**
Rauschbrand 2381....	0	...	0	320	320	640	160	160	...	80	...
Rauschbrand Kitt...	160	...	160	0	0	0	0	80	...	0	...
Rauschbrand Foth..	80	...	320	80	160	0	0	0	...	240	...

1 c.c. antigen used in dilutions of 1:40, 1:80, 1:160, 1:320, 1:640, and 1:960 of serum.

agar. Strains Berg and Foth also have a tendency to ferment salicin. This tendency was emphasized on growing these strains in brain medium containing 2% peptone and containing a trace of iron salts. Strain Reddish is atypical in that it grows in 2% glucose agar and blackens gelatine, thus having some of the characters of C. novyi. Strain Kitt has typical C. oedematis fermentation reactions. Strain 845 in a nongas-producer. The reactions of this strain were not easily obtained, no growth being obtained in a number of trials.

AGGLUTINATION REACTIONS

The agglutination reactions of these strains were studied by the use of brain-liver medium cultures and of anti-serums produced in horses and rabbits. These reactions are shown in table 4. From the

agglutination tests, strain 1, Hall, and Foth are shown to be C. chauvoei strains of the type used to immunize the horses for the production of serum 46, strain Lister, Reddish and Berg types intermediate between C. chauvoei and C. oedematis or a third species. Strain C. oedematis sheep is probably a type 1 strain, 037737 a type 3 strain; strain Kitt is also a type 3 strain; strain 2381, a C. chauvoei, or possibly a Reddish strain.

SUMMARY

A review of previous work indicates that C. chauvoei is the primary cause of blackleg in cattle. C. oedematis and other anaerobes may be associated with it.

Pathogenicity tests show that C. chauvoei is highly pathogenic for cattle, but that injections of C. oedematis and C. novyi are nonpathogenic for these animals.

Filtrates and muscle extracts from cases of blackleg caused by typical C. chauvoei cultures are shown to be nontoxic. C. chauvoei does not produce a soluble exotoxin.

Biologic products made from pure nontoxic strains of C. chauvoei are shown to be highly efficient, while toxic products and those made from atypical products are of little or no value.

Strains obtained from California, Scotland, France, Germany, and Switzerland are shown to be identical with the Kansas type strains. Reports from other workers indicate that the organisms causing La Mancha in Argentina, Manqueira in Brazil and blackleg in South Africa and Australia are also the same.

Four strains of C. chauvoei obtained from different sources are shown to be atypical.

C. chauvoei is shown to be a diffident saccharolytic organism fermenting glucose, lactose, and sucrose under favorable conditions. C. oedematis is seen to be a vigorous saccharolytic organism which ferments all sugars under favorable conditions but under adverse conditions ferments only glucose, lactose, and salicin. C. novyi is shown to blacken unfiltered gelatine.

The agglutination reactions correspond fairly closely with the fermentation reaction tests.

From a study of the pathogenicity tests, the immunization tests, and field use of blackleg aggressin and filtrate produced from typical strains, it is suggested that the inclusion of atypical strains, C. oedematis strains or intermediate types, while being theoretically advisable, would not

increase the efficiency of the pure products to any appreciable extent. The use of about 250,000 doses of blackleg filtrate [28] and of several million doses of blackleg aggressin [15] produced from Kansas type cultures has been followed by a loss of less than 1 in 10,000. These products have been used successfully in all parts of the United States, in Central America, Argentina, and South Africa.

[28] J. Am. Vet. Med. Assn., 1925, 20, p. 632.

THE BACTERIAL FLORA OF MARKET OYSTERS

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In the beginning of December, 1924, an increase in the prevalence of reported cases of typhoid fever in Chicago was noted by the department of health. A report of the epidemiology of 129 verified cases of typhoid fever from Nov. 30, 1924, to Jan. 21, 1925, by Dr. Herman N. Bundesen [1] disclosed the probable source of infection to be contaminated oysters in 69 instances, or 65.1%, of the cases contracted in Chicago.

An independent federal investigation by Lumsden et al., [2] of the typhoid situation in Chicago, New York, Washington and elsewhere, during this period, likewise showed that the main factor in the spread of the infection was, beyond a reasonable doubt, shell oysters from a certain locality.

Many previous epidemics of typhoid fever attributed to the consumption of polluted oysters have been reported in the literature. It has also been noticed that there is usually an increase in gastro-intestinal disturbances other than typhoid fever, in connection with many of these epidemics. Apparently, however, this was not entirely true in the study of the cases of typhoid fever in Chicago. This observation, as will be seen, may have some bearing on the bacterial findings in this paper In view of this reported evidence the presence of other micro-organisms, in addition to B. coli and B. typhosus, may be of significance in judging the sanitary quality of oysters with particular reference to infections like typhoid fever and the intoxication of food poisoning that may follow their consumption.

The subject of the bacterial flora of oysters has been studied and discussed by a number of investigators. The work of Foote,[3] Wood,[4] Fuller,[5] and others indicates that the normal flora of unpolluted oysters corresponds to the flora of the sea water in which they are grown.

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[1] J. Am. Med. Assn., 1925, 84, p. 641.
[2] Public Health Reports, Supplement No. 50, 1925.
[3] Med. News, 1895, 66, p. 320.
[4] Brit. Med. J., 1896, 2, p. 759.
[5] U. S. Bureau of Fisheries Rep., 1904, p. 189.

Houston,[6] Klein,[7] Ferguson,[8] Clark,[9] and Hewlett[10] have determined that oysters free from sewage do not harbor B. coli. Klein,[7] Johnstone,[11] and Stiles[12] have reported the isolation of B. typhosus from oysters. Stiles reports an organism thought to be the source of infection in a typhoid fever outbreak at Goshen, N. Y. In this epidemic there were reported 17 well defined cases of typhoid fever and 83 cases of gastroenteritis. He likewise reported the isolation of a number of typhoid-like organisms from shell fish. His concluding statement in regard to these suspected typhoid organisms is: "These cultures cannot be classified as true types of B. typhosus, although biologically they closely resemble this general group of organisms."

As soon as oysters came under suspicion as the source of infection in the outbreak of typhoid fever in Chicago, an extensive bacteriologic study of oysters collected in the Chicago market was begun. The B. coli score of a greatly increased number of routine samples was determined, and in addition the bacterial flora of each sample was studied in an endeavor to isolate and classify the typhoid-like organisms found in the course of routine examination for B. typhosus.

In the study of the bacterial flora, all organisms which gave colorless colonies by direct plating on Endo's medium were isolated. In the 2 months that the oysters were being examined, 150 such cultures were obtained, and cultural and serologic studies showed 81 organisms of types closely related to the typhoid-dysentry group. Eight strains proved to be B. coli of atypical appearance on Endo's medium. Several strains of B. proteus as well as B. fecalis alkaligenes, B. cloacae and B. fluorescens were identified. A group of 15 organisms was isolated which closely resembled the paratyphoid group. One organism culturally identical with Flexner's dystentery bacillus was found. In no instance was B. typhosus isolated.

GENERAL TECHNIC

The B. coli score was determined by the standard methods of the American Public Health Association as published in the journal of the association in July, 1922.

The isolation of typhoid-like colonies was accomplished by inoculating the surface of Endo's medium with the crushed meats of the

[6] Royal Sewage Commission, 4th Rep., 1904, p. 220.
[7] Medical Office, Local Gov't Board of Gt. Britain, Rep. 24, 1874-5, Appendix 2, p. 109.
[8] Virginia Health Bull., No. 11, 1909.
[9] Rep. State Board of Health of Mass., 1905, p. 449.
[10] Brit. Med. J., 1903, 1, p. 1082.
[11] J. Hyg., 1909, 9, p. 433.
[12] U. S. Dept. of Agriculture, Bureau of Chemistry Bull., No. 156, 1912: No. 136, 1911.

oysters. Colorless colonies on the surface of the Endo medium were purified by seeding in fluid Endo's agar at 45 C., and pouring plates for the deep colony method of isolation. The typhoid-like colonies on these plates were transferred to triple sugar (sucrose 1%, dextrose 1% and lactose 1%) agar slants in which Andrade's indicator was used to show the change in reaction. The organisms which failed to show a typical B. coli reaction in these slants in 24 hours at 37 C., viz., gas in the butt of the tube and acidity throughout the medium, were further cultivated in tryptophane water [13] for 5 days at 37 C., to determine indol production [14] by stab inoculation in 14% gelatin for 30 days at 20 C. for proteolytic enzymes and on plant agar slant for stock.

In addition, the fermentative reactions were studied by inoculation in triplicate of litmus milk and various carbohydrate mediums, the basic bouillon or agar of which had a reaction of P_H 6.8. The sugar solutions were sterilized by filtration, and these were added to the basic medium to make a concentration of 2%. The sterility of the sugar mediums was assured by incubation at 37 C. for 24 hours before use. All carbohydrate broth mediums were inoculated with 0.25 c c., of a 10 c c., physiologic salt suspension of a 24-hour agar slant growth of the suspected organisms. The reactions were read by means of brom-cresol purple as indicator after 5 days' incubation at 37 C. An effort was made to identify by agglutination tests those organisms whose cultural and morphologic characteristics warranted their tentative classification in the typhoid-paratyphoid group.

COLON SCORE

The B. coli scores of shell and shucked oysters examined in the laboratory during the 1924-1925 oyster season were less than 50 in about 65% of the shucked oysters and 98 per cent. of the shell oysters. The predominance of low scores was also noted in the oyster samples from which suggestive colonies were isolated for identification and classification.

Apparently the information herein obtained from the score method of determining the sanitary quality of oysters, particularly those in the shell, is somewhat in doubt as to its value. Evidently a standard of 50 can easily be met by nearly all shell oysters and the majority of shucked oysters. Perhaps two standards, one for shell and one for

[13] Standard Methods of Water Analysis, Am. Public Health Assn., 1923.

[14] Malone and Goré, cited by Levine, Agivna, and Weldin: Am. Jour. Pub. Health, 1925. 15, p. 18.

shucked, are necessary, with the standard for the former less and the standard for the latter greater. In the event that oysters may be chemically treated to insure purity, a third standard even less than 50 may be easily met. The practicability of the colon score as a determining factor of sanitary quality of oysters, especially if tests are made at distant points from the base of supply, is at present argumentative.

TABLE 1

B. Coli Scores of Market Oysters During 1924-1925 Season

Month	Total Samples	No B. Coli Found		B. Coli Score 1:50		B. Coli Score 140:500	
		Number	%	Number	%	Number	%
Shucked Oysters:							
September	19	2	10.5	0	....	17	89.5
October	49	5	10.2	4	8.2	40	81.8
November	5	2	....	1	....	2	....
December	199	14	7.0	82	41.2	103	51.7
January	255	40	15.7	146	57.2	69	27.1
February	121	40	33.1	73	60.3	8	6.6
March	146	13	9.6	91	61.5	42	21.9
April	63	14	22.2	33	52.4	16	25.4
	857	130	15.1	430	50.2	297	34.6
Shell Oysters:							
December	148	50	33.8	93	62.8	5	3.2
January	48	36	75.0	12	30.7	0	....
February	47	30	63.8	17	31.8	0	....
March	50	23	46.0	27	54.0	0	....
April	35	14	40.0	21	60.0	0	....
	328	153	46.6	170	51.8	5	1.5

TABLE 2

B. Coli Scores on Oysters Examined in Which Suggestive Organisms Were Found

Sample	Count 1:50		Count 140 and Above		Total No.
	No.	%	No.	%	
Shucked	40	57.2	30	42.8	70
Shell	6	100	0	0	6

A preliminary test for fermentative activity of the suspected organisms was made in broth containing the following sugars: dextrose, mannitol, maltose, lactose, xylose, rhamnose and dulcitol. The reactions in these mediums yielded data sufficient to identify the following organisms: B. alkaligenes, B. cloacae, B. acidi lactici, B. aerogenes, B. proteus groups and 8 strains of B. coli atypical in their reaction on Endo's medium. There remained a group of unidentified organisms with cultural reactions similar to the paratyphoid group.

A macroscopic agglutination test using the latter organisms as antigens was made in a series of dilutions up to 1 : 3,200 with standardized antityphoid and antiparatyphoid commercial serums. A suggestive agglutination with the antityphoid serum was obtained with some of the strains. This agglutination was not present above 1 : 500 dilution of the serum, which reaction was obviously within the cross agglutination power of the high titer serum used. The B. typhosus controls were agglutinated completely in much higher dilutions, viz., up to 1 : 6,000 and partially in dilutions up to 1 : 12,000.

The fermentative reactions of this group of organisms were rechecked, using dextrose, lactose and sucrose broths and litmus milk. The organisms which produced acid and gas in dextrose broth and which

TABLE 3

TABLE OF CROSS AGGLUTINATIONS (MACROSCOPIC)

Organisms	Laboratory No.	B. Typhosus Antiserum			B. Paratyphosus Antiserum	
		Chicago Health Dept.	Commercial X	Commercial Y	Alpha	Beta
		Complete Agglutination in Dilutions of				
B. proteus	5849	.....	1:100	1:50	.....	.....
B. proteus	5971	.....	1:100	1:20	.....	.....
B. proteus	5756	1:200	1:400	1:50	1:50	1:50
Unidentified	5937	.....	1:200	1:50	.....	.....
B. coli	5887	.....	1:100	.....	.....	.....
B. acidi lactici	5992	.....	1:100	1:50	.....	.....
B. aerogenes	5997	.....	1:100	1:50	.....	.....
B. paratyphosus ? control	413W	1:400	1:400	1:400	1:200	1:200
B. typhosus Rawlings	....	1:3,200	1:3,200	1:400	1:100	1:400
B. typhosus Hopkins	....	1:3,200	1:1,600	1:200	.....	1:100
B. paratyphosus A	....	.....	.....	.....	1:3,200	1:200
B. paratyphosus B	....	.....	.....	.....	1:400	1:3,200

failed to do so in lactose and sucrose broths were tested for fermentative activity in rhamnose, sorbitol, raffinose, dextrin, salicin and xylose broths. The strains producing acid and gas in sorbitol and xylose were retested by the agglutination method, using a standardized commercial antityphoid serum and a serum prepared by one of us in which the Rawling's strain of B. typhosus was used. The agglutination tests were negative with both serums. Further identification tests were made by inoculating arabinose, dulcitol, trehalose and inosite broths with the unknown organisms. They are more closely related to the paratyphoid B group than to the typhoid group in their fermentation reactions because they produced acid and gas in rhamnose, with the exception that some strains failed to ferment sorbitol.

TABLE 4

PRELIMINARY TESTS OF FERMENTATIVE REACTIONS

Organisms Identified	Gelatin	Indol	Triple Sugar		Carbohydrates								Litmus Milk	Gram Stain	Motility	Laboratory No.
			Slant	Butt	Dextrose	Mannitol	Maltose	Lactose	Xylose	Rhamnose	Sucrose	Dulcitol				
B. faecalis alkaligenes	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	387, 5931, Indiana I, Indiana V, 6054
B. cloacae	+	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	5967
B. acidi lactici	—	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	—	⊕	+	—	—	5992, 230
B. aerogenes	—	—	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	40, 91A, 5997
B. coli	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	+	—	+	5853
	—	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	—	+	—	+	5887
	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	6067
	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	+	—	+	166
	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	39
	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	359
	—	+	+	⊕	⊕	⊕	⊕	⊕	—	⊕	⊕	+	+	—	+	187
	—	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	—	+	+	—	+	112
B. proteus I	+	+	+	⊕	⊕	⊕	⊕	—	⊕	⊕	⊕	⊕	+	—	+	115B, 255, 204, 476, 602A, 653, 5849, 5868, 5756, 115W
B. proteus II	+	—	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	436, 232, 5832, 5846, 5971, 406
B. proteus III	+	+	+	+	+	+	—	—	—	—	+	—	+	—	+	495, 577, 604, 624, 627, 629, and 128
B. proteus IV	+	+	—	+	+	—	+	—	+	—	—	—	+	—	+	6019, 6130, 128 and 349
B. proteus V	+	+	—	⊕	⊕	⊕	⊕	—	⊕	—	⊕	⊕	+	—	+	155W, 146A, 161A and 324
B. proteus VI	+	+	—	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	155B and 6018
B. proteus VII	+	+	+	+	+	+	—	—	—	—	—	—	+	—	+	41A and 237
B. proteus VIII	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	322, 62 and 116
B. proteus IX	+	—	—	+	⊕	⊕	⊕	—	—	—	—	—	+	—	+	634
B. proteus X	+	—	—	⊕	+	—	—	—	⊕	—	⊕	⊕	+	—	+	146B, 61B and 293
B. proteus XI	+	—	—	⊕	⊕	⊕	⊕	—	—	—	—	—	+	—	+	308
B. proteus XII	+	—	—	⊕	⊕	⊕	⊕	—	⊕	—	⊕	⊕	+	—	+	295

+ indicates acid production or positive reaction; ⊕, acid and gas production; —, negative to reaction.

TABLE 5

FERMENTATIVE REACTIONS OF UNIDENTIFIED ORGANISMS

Laboratory No.	Gelatin	Indol	Triple Sugar		Carbohydrates															Litmus Milk	Gram Stain	Motility	
			Slant	Butt	Dextrose	Mannitol	Maltose	Lactose	Xylose	Rhamnose	Sucrose	Dulcitol	Dextrin	Inosite	Trehalose	Raffinose	Salicin	Sorbitol	Arabinose				
435	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	—	—	—	⊕	+	—	+	Paratyphoid-like organisms
362	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	+	—	+	
302	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
251	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
409	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	⊕	⊕	⊕	—	—	⊕	+	—	+	
413W	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	Alk.	—	+	
346	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
253	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	⊕	⊕	⊕	—	—	⊕	Alk.	—	+	
335	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
410	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	+	—	+	
333	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	+	—	+	
230j	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	+	—	+	
271	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
138B	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
138A	—	—	—	+	⊕	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	⊕	⊕	—	—	⊕	+	—	+	
41311	—	—	—	+	+	+	+	—	+	+	—	+	+	+	+	+	+	+	+	+	—	+	Flexner's B. dysenteriae

+ indicates acid production or positive reaction; ⊕, acid and gas production present; —, negative to reaction; Alk., alkaline reaction.

An attempt was made to agglutinate these 15 strains with serums of B. paratyphosus A, B. paratyphosus B, B. suipestifer and B. enteriditis. All aggluination tests were negative. These organisms are similar culturally to B. paratyphosus B, differing culturally from the latter organism in the production of acid and gas in raffinose and the failure to produce acid in inosite and sorbitol broths.

In the course of the study one organism was isolated which was identical culturally with the Flexner type of B. dysenteriae, but it failed to agglutinate with either the Flexner or Shiga dysentery antiserums.

COMMENT

Epidemiologic analyses by several investigators working independently indicate conclusively that the consumption of oysters in the latter part of 1924 was the direct cause of a large number of cases of typhoid fever. The condition of this large industry for that period must have been similar to that of other years, and the focusing of attention on the increased incidence of typhoid fever should be credited to the vigilance of the several health authorities concerned. The bacteriologic findings quoted in this article stress the fact that food poisoning outbreaks of heretofore unknown origin might have been due to oysters. Certainly, it is most interesting to discover certain organisms present in the bacterial flora of oysters that are so obviously closely allied culturally to the paratyphoid group. Of far more importance to public health workers in the laboratory are the cross-agglutinations with specific antiserums, particularly B. typhosus. Undoubtedly, the incidence of typhoid fever in the early fall months or the period preceding the hibernation of oysters will be carefully studied, as raw oysters cannot be excluded as an epidemiologic possibility, particularly in large cities.

SUMMARY

No B. typhosus was found in 784 samples of shucked and 328 samples of shell oysters examined at the time of the recent epidemic in Chicago.

Several organisms were found which were closely related to the members of the paratyphoid-dysentery group. We were not able to classify these organisms definitely, however, in this group by all cultural or specific serologic reactions.

It is of interest to note that some of these organisms were agglutinated completely in a dilution of 1 : 400 by an antityphoid serum of high titer.

EXPERIMENTS ON THE PURIFICATION OF CULTURES OF SPIROCHAETA PALLIDA BY CHEMICAL METHODS

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The present communication is one of a series of studies from this laboratory on the development of improved methods of isolating and cultivating Spirochaeta pallida. The difficulties inherent in the older methods and an analysis of the requirements of a satisfactory culture medium have already been discussed.[1] In this paper, we wish to record the results of experiments with the use of various germicidal substances, which were selected with the hope of discovering chemicals that would have a selective action in inhibiting the growth of contaminating bacteria, and not affect the viability of Spirochaeta pallida.

REVIEW OF THE LITERATURE

The phenomenon of the selective inhibitory action of dyes on bacterial development has been of interest to bacteriologists ever since the discovery of aniline dyes. Among the earliest workers on this subject were Rozsahegyi,[2] Shillings[3] and Koch.[4] They showed that various organisms, among them the tubercle bacillus, are inhibited in their growth, or killed by dilutions of various dyes. Bacteria were found to differ in their susceptibilities to a definite dyestuff, and dyestuffs to manifest enormous variations in effect when used against groups of bacteria.

As is well known, Conradi and Drigalski,[5] in 1902, first incorporated crystal violet into mediums for the isolation of typhoid, paratyphoid and colon bacilli. Brilliant green was employed in fluid medium by Browning, Gilmore and Mackie[6] to suppress the colon bacillus in a mixed culture of colon and typhoid. The numerous contributions of Churchman and his collaborators[7] dealing with the bacteriostatic and bactericidal action of various dyes toward gram-negative and gram-positive organisms have established the selective action of gentian violet toward the gram-positive bacteria and that of acid fuchsin toward the gram-negative. Extending his work to include other dyes, Churchman was able to formulate his theories of "intrinsic" and "extrinsic" bacteriostasis. To the bacteriostasis a substance exhibits when bacteria are exposed to it before being planted on appropriate medium, he gave the name "intrinsic bacteriostasis," while "extrinsic bacteriostasis" is that which a substance exhibits when

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[1] Weiss, C., and Wilkes-Weiss, D.: Jour. Infect. Dis., 1924, 34, p. 212.
[2] Centralbl. f. Bakteriol., I. O., 1887, 2, p. 418.
[3] Ibid., 1890, 8, p. 155.
[4] Tr. 10th Int. Med. Congress, 1890, p. 564.
[5] Ztschr. f. Hyg. u. Infectionskrankh., 1902, 39, p. 283.
[6] Jour. Path. & Bacteriol., 1913-4, 18, p. 146.
[7] Proc. Soc. Exper. Med. & Biol., 1912, 16, p. 221; ibid., 1922, 20, p. 19; Jour. Exper. Med., 1921, 33, 569; ibid., 1923, 37, pp. 1, 543.

it is present in the medium in which the unstained bacteria are to be planted. The paralysis of the reproductive mechanism of the organism without otherwise injuring the bacteria was termed "genistasis." In Churchman's experiments, the selective behavior of gentian violet and allied dyes was found to be the same, whether incorporated in the medium and then tested, or added directly to the bacteria and later inoculated on the plain medium. This, however, does not always hold true in the acid dyes, as in acid fuchsin and neutral proflavine. Among the drugs exhibiting a reverse selective activity (having the power to kill gram-negative but sparing the gram-positive organisms) were included sulphanilic acid, chromotropic acid, sodium ethyl-sulphonate and phenol sulphanate as well as acid fuchsin. This reverse selective activity Churchman attributes to the presence of the SO_3 group.

The action of a few chemicals on spirochetes has already been studied by Bronfenbrenner and Noguchi.[8] They showed that alcohol in a dilution of 1 : 10 did not kill, while small quantities of phenol, tricresol, and mercuric chloride, far below the sterilizing limits of these antiseptics, exerted a marked stimulating action on the growth of the organisms after one hour's exposure. Mercuric chloride did not kill the spirochetes in a dilution of 1 : 200,000, whereas 1 : 16,000 proved lethal to the colon (after contact of 1 hour at 37 C.). Akatsu[9] tested the resistance of spirochetes to the action of hexamethylenetetramine derivatives and mercurial compounds. His list includes phenol which did not prove spirocheticidal in a dilution of 1 : 5,000. Formalin 1 : 1,000, lysol 1 : 7,500, and arsphenamin 1 : 10,000, etc., also failed to kill spirochetes.

Uhlenhuth and Mulzer[10] carried out experiments on the effect of various chemicals on spirochetes of fowls (Spirochaeta gallinarum). They worked with a testicular emulsion of the former and made dark-field examinations after the drug had acted for varying periods of time. However, no cultural experiments were made. Antiformin in low dilutions dissolved or destroyed the spirochetes; in higher dilutions, however, it had little or no effect. The motility of the organism was lessened or rendered nil with the use of certain strengths of mercuric chloride, ether, formalin, Lugol's solution, phenol, glycerol and the hydroxides of sodium and potassium.

In carefully going over the literature on Spirochaeta pallida, we find only isolated and fragmentary attempts at the use of chemicals with the purpose in mind of purifying the cultures. Sowade[11] claimed that 70% alcohol was at times efficacious in rendering a first generation of spirochetes in culture medium comparatively pure. Proca, Danila and Stroe[12] incorporated alkaline pyragallol and gentian violet in their medium for the cultivation of Spirochaeta pallida, thus making use of Churchman's theory of extrinsic bacteriostasis referred to above.

METHOD OF INVESTIGATION

Before selecting the technic suitable for this work, it was necessary for us to investigate the question of "intrinsic" versus "extrinsic"

[8] Jour. Pharmacol. & Exper. Therap., 1912-3, 4, p. 333.
[9] Jour. Exper. Med., 1917, 25, p. 363.
[10] Deutsch. med. Wchnschr., 1911, 37, p. 713.
[11] Deutsch. med. Wchnschr., 1912, 38, p. 796.
[12] Compt. rend. Soc. de biol., 1912, 73, p. 235.

bacteriostasis. The latter method, as stated, implies planting the organism in a medium in which the germicide has already been incorporated. We made many tests with this method, but finally abandoned it, because we found that while most of the germicidal power of dyes is lost in this way (due in part to the absorption of the dye by the proteins of the medium), some still remains, and being permitted to act over a prolonged period, it eventually causes the death of our delicate organisms. Hence the method of "intrinsic" bacteriostasis, which is the bacteriostasis a substance exhibits when bacteria are exposed to it before planting on to appropriate mediums, was used.

A chosen dilution of the germicide was placed in a small sterile test tube. This was combined with an equal quantity of Spirochaeta pallida culture, grown on modified Schereschewsky medium. A dark-field examination revealed from 40 to 75 active spirochetes per field. One drop of a 24 hour growth of either Staph. albus or B. coli was added, and the tube was placed in a water bath at 37.5 C. At definite intervals of 1, 5, 15 and 60 minutes, portions were transferred to tubes of gelatinized horse serum.

The medium used was composed of horse serum (1 part), "hormone" broth P_H 7.6 (4 parts), distributed in spirochete tubes, 20 x 1.5 cm., and heated slowly to 80 C. The tubes were kept at 80 C. for ½ hour and while still hot were covered with a layer of melted sterile petrolatum, 1 cm. high.

The planted tubes were incubated at 37.5 C., and the first examination for spirochetal growth and bacterial contamination was made after 7 days. Growth was judged by dark-field illumination (Leitz-Wetzlar oil immersion, 1/12 objective) and subsequent replanting on fresh stock medium. Seeding of the material on agar slants by the stroke method gave evidence, after 48 hours incubation as to the presence or absence of bacteria. The possibility that certain chemicals might exert a bacteriostatic activity and not be bactericidal was kept in mind, and in several instances reexaminations were made (under the dark field and by the stroke agar method) of the tubes which had previously shown freedom from the contaminating organisms at stated intervals. For the purpose of testing mixed contaminations, one small drop of a combination of staphylococcus and colon cultures was used.

The dyes, with the exception of Grübler's gentian violet and methylene blue, were obtained from Coleman and Bell. Each dye or medicament was prepared in a 1% solution (unless lower dilutions were

required) in sterile distilled water or normal salt solution. Higher dilutions were prepared from these, using the same diluent that had been used in the making of the stock 1% solution.

SUMMARY OF RESULTS

Of the 17 chemicals tested against the staphylococcus, 14 proved satisfactory under proper conditions. The remaining 3 chemicals either destroyed the spirochetes or did not manifest a bactericidal action against the coccus. Brilliant green, in a strength of 1 : 500 killed the bacteria in 1 minute, but at the same time interfered with the growth of the

TABLE 1

SELECTIVE INHIBITORY ACTION OF VARIOUS GERMICIDAL SUBSTANCES ON THE MIXED GROWTH OF SPIROCHAETA PALLIDA AND STAPHYLOCOCCI

Nature of Drug	Name of Drug	Dilution *	Time of Exposure Recommended, Minutes
Dyes	Acid fuchsin	1:200	1
		1:1000	5
	Gentian violet	1:10,000	1
		1:100,000	1
	Mercurochrome	1:1000	1
	Mercurophen	1:11,000	1
	Methylene blue	1:500	1
Arsenicals	Monarsone	1:10	1
		1:20	15
	Neo-arsphenamine	1:1000	1
	Atoxyl	1:100	5
	Acid arsphenamine	1:1000	1
Chlorine compounds	Antiformin	1:150	1
		1:175	5
General germicides	Lugol's solution	1:2	1
Special	Ethyl hydrocuprein hydrochloride	1:1000	5
Reducing substances	Formaldehyde	1:500	1
Silver compounds	Neosilvol	1:100	1

* The dilution and time exposures tabulated indicate the strength of substance and maximum contact necessary to be germicidal toward the staphylococcus and yet exert no deleterious effect on the spirochetes.

spirochetes. In a dilution of 1 : 3,000 for 1 minute, the dye exerted no effect on the bacteria and still injured the spirochetes. An undiluted solution of peroxide of hydrogen acted similarly, while a strength of 1 : 2 permitted growth of the coccus. Pyronin 1 : 20,000, killed the bacteria after an exposure of 60 minutes, but with deleterious action on the spirochetes.

Realizing that the gram-negative organisms are more resistant to the action of germicides, we directed more of our attention to finding a gram-negative bactericide. We hoped, as did Burke and Skinner,[13] that those chemicals which would destroy the colon would surely kill the staphylo-

[13] Jour. Exper. Med., 1924, 39, p. 613.

coccus. These experiments we undertook with misgivings, for we knew that spirochetes are from 20 to 100 times as sensitive to the action of drugs as the bacillus (Bronfenbrenner and Noguchi [8]), and we were justly afraid that those germicides which would prove gram-negative bactericides would also be spirocheticidal. In this connection, we definitely substantiated [14] the fact that the Spirochaeta pallida is itself a gram-negative organism. In all, 50 drugs were tested, 10 of which were dyes. Of the 10 dyes, only 4 had the power of killing the colon bacillus: crystal violet, 1 : 100, acting for 60 minutes; malachite green, 1 : 3,000, for 15 minutes; acriflavine, 1 : 200, for 1 hour, and mercurophen, 1 : 200, for 5 minutes. Twelve of the 40 chemicals (excluding the dyes) proved bactericidal in certain dilutions: neosilvol, 1 : 10, in 5 minutes; silver nitrate, 1 : 100, in 15 minutes; tricresol, 1 : 100, in 1 minute; mercuric chlorid, 1 : 100, in 1 minute; trichloracetic acid in 15 minutes; undiluted butylic alcohol in 60 minutes; a 2% iodine solution in 1 minute; formaldehyde, 1 : 10, in 1 minute and 1 : 20 in 5 minutes, respectively; phosphotungstic acid (Folin's reagent) in 1 minute; thorium chloride 1 : 100 in 1 hour; selenium oxychloride, 1 : 100, in 1 minute and 1 : 1,000 cadmium iodide in 60 minutes. Listing in a similar manner those chemicals and dyes manifesting a spirocheticidal activity, there were 7 of the 10 dyes which either destroyed or caused a poor growth of the organism with an exposure of 1 minute.

1. Crystal violet, 1 : 100.
2. Methylene blue, 1 : 25.
3. Acriflavine, 1 : 100.
4. Malachite green, 1 : 3,000.
5. Gentian violet, 1 : 30.
6. Mercurochrome, 1 : 100.
7. Mercurophen, 1 : 200.

Little or no spirochetal growth resulted after a 1 minute exposure to the following 27 drugs:

Atoxyl	Butylic alcohol	Antimony tartrate
Monarsone	Lugol's solution	Ammonium molydate
Acid arsphenamine	Silver nitrate	Cadmium iodide
Antiformin	Ethylhydrocuprein hydrochloride	Chromotropic acid
Neosilvol	Phenol	2% Iodine solution
Strontium nitrate	Mercuric chloride	Formaldehyde
Aluminum sulphate	Thorium chloride	Phosphotungstic acid
Telluric acid	Lanthanium nitrate	Sodium tartrobismuthate
Cobalt nitrate		Vanadium chloride
Sodium fluoride		

[14] Wilkes-Weiss, D.: Jour. Lab. & Clin. Med., 1924, 9, p. 716.

But the fact must not be lost sight of that the real purpose of our experiments was to find a chemical which would prove bactericidal and at the same time not spirocheticidal in a given concentration and interval of contact. Among the 50 chemicals tested, we were able to find only 4 which complied with the requirements. Tricresol 1 : 100 killed the colon bacillus in 1 minute, but produced a condition of genistasis in spirochetes. A 1 : 100 dilution of trichloracetic acid in 15 minutes produced a fair growth of spirochetes uncontaminated by the colon. Formaldehyde 1 : 20 [15] was bactericidal in 5 minutes with a genistatic effect on the parasite, i. e., a paralysis of the reproductive mechanism. And, finally, selenium oxychloride 1 : 100 proved an excellent bacillide in one minute. The spirochetes themselves underwent slight morphologic changes. A fairly good growth was obtained on subsequent subcultures.

COMMENT

It will be seen from the data presented that many antiseptics exert marked bactericidal or bacteriostatic action on Staph. albus, with very little effect on the growth of B. coli. Antiformin, for example, which in a dilution of 1 : 150 killed staphylococci in 1 minute, had no bacteriostatic action against B. coli in as high a concentration as 1 : 15 for 15 minutes. Neo-arsphenamine showed a similar selective activity against the gram-positive organism in a dilution of 1 : 1,000 with a slight stimulating effect on the spirochetes themselves; whereas, in the same strength after 15 minutes' contact, it allowed a luxurious growth of the colon bacillus.

The spirochetes and the staphylococci were affected by copper sulphate, 1 : 10, the former showing very poor growth with degenerative changes, the albus being destroyed. Optochin or ethylhydrocuprein hydrochloride, introduced by Morgenroth and Levy, a drug possessing high pneumococcidal power, proved itself to be staphylococcidal in a dilution of 1 : 100, with an accompanying inhibitory action on the spirochetes. The growth of the colon bacillus was not delayed or affected in any way by the drug in a 1 : 1,000 solution. Glycerol, in full strength, exerted no harmful action either on the spirochetes or on the bacteria. This confirms the findings of Metchnikoff.[16]

The newer mercury compounds, mercurochrome and mercurophen were also tested. Mercurophen [17] in a dilution of 1 : 11,000 freed the

[15] It must be remembered in interpreting our results that the final dilutions of the chemical is twice that indicated in the tables, for equal parts of spirochete emulsion and germicide were always used.

[16] Quoted by Sobernheim in Kolle u. Wassermann: Handbuch der Path. Mikro-organismen, 1913, 7. p. 745.

[17] Schamberg, Kolmer and Raiziss: Jour. Infect. Dis., 1919, 24, p. 547.

spirochetes from staphylococci with no untoward effect on the former, even in a concentration of 1:600. Our experiments with mercurochrome show that although B. coli was not affected in a 1:100 dilution, the drug was lethal to the staphylococci in 1:1,000 after one minute's exposure, at the same time giving a good spirochetal growth.

We were disappointed to find that the two dyes, acriflavine and acid fuchsin, which were recently recommended by Churchman as gram-negative germicides, did not fulfil our purpose. Our results are in concord with those of Gay and Morrison [18] in their work on streptococcus empyema, in which they found that there was a failure of the dyestuff to act in the pleural cavity, where there is a condition of negative atmospheric pressure. Egerth [19] called our attention to the fact that heating serum to the temperature employed in making Schereschewsky's medium will destroy its power to enhance the germicidal power of acriflavine. Gentian violet proved a most satisfactory gram-positive bactericide in as high a dilution (in saline) as 1:100,000, and at the same time, a stimulant to the growth of the pallidum. Acting on the suggestion made by Churchman in his paper on "Bacteriostasis of Mixed Dyes," a combination of gentian violet, 1:100,000 and acriflavine 1:1,000, was allowed to act on spirochetes contaminated with staphylococci and colon bacilli. The latter survived even after an exposure of 60 minutes, an interval which was sufficient to kill the spirochetes.

When an alcoholic solution of the dye, gentian violet, was used against staphylococcus and spirochetes, both organisms suffered. On further experimentation, we found that 70%, 50%, and absolute alcohol, all proved lethal, both to the spirochetes and to the staphylococci. Brilliant green, a dye most successfully used in the isolation of the typhoid bacillus, was little tolerated by the Sp. pallida in the dilutions in which it proved to be a good gram-positive bactericide. Methylene blue, in a dilution as low as 1:100 did not affect B. coli or the spirochetes, but it killed staphylococci in as high a concentration as 1:10,000 after one minute's contact. In recent researches by Krantz,[20] methylene blue was incorporated in the medium for the cultivation of the spirochetes as an index of the oxygen tension of the medium.

As Ehrlich [21] and Uhlenhuth, Gross and Bickel [22] found atoxyl to be nonspirochetal in vitro, we included this chemical (the sodium salt of

[18] Jour. Infect. Dis., 1921, 28, p. 1.
[19] Personal communication of unpublished data.
[20] Berl. klin. Wchnschr., 1923, 2, p. 1698.
[21] Quoted in Kolle u. von Wassermann: Handbuch der Path. Mikro-organismen, 1913, 7, p. 745.
[22] Deutsch. med. Wchnschr., 1907, 33, p. 129.

p-aminophenyl arsinic acid), in our experiments. The salt in a dilution of 1 : 1,000 showed no adverse affect on our spirochetes, but rendered the culture free from staphylococcus. The colon bacilli were unaffected even when exposed to as low a dilution as 1 : 50, this strength allowing little or no growth of the spirochetes.

Our results confirm Kligler's finding's [23] that simple aniline derivatives and dyes, as well as many other chemicals, are more toxic for gram-positive than for gram-negative organisms. Akatsu's observations [9] with arsphenamin, phenol and formalin (which he found to be non-spirocheticidal) tally with our own. Contrary to the findings of Uhlenhuth and Mulzer,[10] and more nearly in accord with those of Metchnikoff,[16] glycerol (undiluted), exerted no deleterious action on the spirochetes.

During the course of these experiments, we followed the degenerative changes taking place in Spirochaeta pallida when acted on by germicides. The first evidence of the unfavorable action of the drugs, is a momentary increased activity and motility. This is followed by a decrease in number and an irregularity in the curves of the organisms. The activity diminishes, the locomotion becomes sluggish and the curves become straight lines. No division is seen in the late stages. Dissolution and lysis occur in some, and it is interesting to observe mere skeletons of spirochetes swimming together with exceptionally long spirochetes, wavy rather than cork skrew in appearance. Instead of from 5 to 10 or more curves, there may be from 1 to 3. Granulation often appears in the long axis of the organism, looking like so many dots linked together by a common, but very faint, envelop. In many instances, however, complete absence of motility with no change in morphology is the only microscopic evidence of nonviability in the spirochetes. On subcultivating, the second generation reveals perhaps 1 or 2, sometimes more, per field, of morphologically deteriorated spirochetes, probably the fruit of an occasional spirochete which had escaped complete dissolution and death in the original tube.

Gonder [24] found that spirochetes, especially treponema, offer a great deal of resistance at the beginning of exposure, but finally undergo lysis, their cell bodies becoming swollen or macerated. In the experiments of Akatsu and Noguchi [9] cytolysis occurred in the higher concentrations of the drug. The organisms were also killed, however, without gross destruction of the cells.

[23] Jour. Exper. Med., 1918, 27, p. 463.
[24] Spironemacea in von Prowazek, S.: Handbuch der Path. Protozoen, 1914, p. 671.

SUMMARY

Experiments were undertaken to determine the selective inhibitory action of various germicidal substances on the growth of bacteria which may contaminate cultures of Spirochaeta pallida.

The following chemicals are satisfactory (in the dilution and time of exposure stated) for the purpose of destroying B. coli as well as Staph. aureus without affecting the viability of the reproductive power of Spirochaeta pallida: Selenium oxychloride or tricresol (in a 1 : 100 dilution, to be used for 1 minute); trichloracetic acid (1 : 100 for 15 minutes), or formaldehyde (1 :20 for 5 minutes).

When it is desired to destroy staphylococci alone, a larger variety of chemicals may be employed: Gentian violet, acid fuchsin, mercurochrome, mercurophen, methylene blue, monarsone, neo-arsphenamine, atoxyl, acid arsphenamine, antiformin, Lugol's solution (iodine), ethylhydrocuprein hydrochloride or neosilvol.

WASSERMANN REACTION IN SYPHILIS OF RABBITS

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Observations have been made over a period of several months on the Wassermann reaction in rabbits inoculated with the same strain of Spirochaeta pallida. The original strain was obtained from an inoculated rabbit that was sent by Dr. Louise Pearce from the Rockefeller Institute and is known as number 4,275. Three rabbits of the series were inoculated from rabbit 4,275 and the others from rabbits with later infections.

All the rabbits had well developed scrotal lesions from 1 to 3 months after inoculation and in each lesion spirochetes were found by dark-field examination.

The Wassermann test was made at intervals of approximately one month. Cholesterolized alcoholic extract and acetone-insoluble antigens were used with the antisheep hemolytic system and icebox fixation.

Although the scrotal lesions developed regularly, the Wassermann reactions were rather inconsistent. About half the rabbits had a somewhat typical course in which the Wassermann test became strongly positive about 2 months after inoculation, persisted positive for about 2 months and then became negative, with a later return in some of a low degree positive reaction. None of the rabbits had any visible lesions outside of the scrotum and testicles, with the exception of 4,275, which had a persistent keratitis.

The rabbits with atypical reactions frequently gave a lower degree positive test and of shorter duration. The reactions were irregular as to time of appearance after inoculation, and in one rabbit there was a negative test at every examination.

The scrotal lesions usually healed spontaneously in from 1 to 2 months, and the rapidity of healing without treatment indicates that careful controls must be made in the evaluation of new drugs by their effects on the lesions of rabbits.

The results also would seem to indicate that in rabbits there is a specific reaction which limits the active course of the disease, and that

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Results of Wassermann Tests on Rabbits Inoculated with Spirocheta Pallida

Rabbits	Number of Days After Inoculation Until Initial Lesion and Positive Dark-Field Examination	Number of Days After Inoculation with Corresponding Wassermann Results																						
4275	28	53	87	103	111	151	203	223	231	242	268	303	318	334	355	378	401	426	447	471	.....	515	539	593
		++++	++++	++++	++++	+++	0	±	0	0	0	0	0	0	0	0	0	±	0	0		++	±	0
1	31	.....	34	50	58	98	150	170																
			0	++++	++++	++++	++++	Died																
2	45	.....	34	50	58	98	150	170	178	189	215	250	265	281	302	325	348	373	394	418	449	462	486	540
			0	++++	++++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	33	.....	.....	.....	14	66	76	126	134	145	171	206												
					0	+++	+++	+++	+	0	0	Died												
4	38	.....	.....	.....	.....	.....	.....	19	27	38	64	99	114	130	157	174	197	222	243	407	298	311	335	389
								0	0	0	0	0	0	0	0	0	0	0	0	0	0	±	0	0
5	29	.....	.....	.....	.....	.....	.....	19	27	38	64	99	114											
								0	0	+	++++	+++	Died											
6	31	.....	.....	.....	.....	.....	.....	19	27	38	64	99	114	130	157	174	197	222	243	267	298	311	335	389
								0	0	0	++++	+	0	0	0	0	0	0	0	0	0	0	0	0
9	30	.....	.....	.....	.....	.....	.....	11	27	38	64	91	106	122	149	166	189	214	235	259	290	303	327	381
								0	0	0	0	0	++++	++++	0	0	0	0	0	0	0	0	0	+
10	16	.....	.....	.....	.....	.....	.....	.....	19	30	56	91												
									0	0	0	Died												
11	46	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	35	50	66	87	110	133	158	178	203	234	247	271	325
												0	0	+++	0	0	0	0	0	0	0	++	0	+ —
12	49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	35	50	66	87	110	133	158	179	203	234	247	271	325
												0	0	+	0	0	0	0	0	0	0	Anti-comp.	0	+ —
15	38	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	8	38	54	79	96	134	155	187	...	241
															0	0	0	0	++	+++	++++	++		+
16	40	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	15	31	52	75	98	123	144	155	199	212	236	290
													0	0	0	0	0	0	0	0	0	Anti-comp.	0	0
23*	38	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	15	38	63	84	95	139		152	176	230
															0	0	0	0	0	0		0	±	+++

this reaction is not always of such degree as to produce sufficient serologic changes to make a strong positive Wassermann test.

Pearce and Brown [1] state that spirochetes can always be recovered from the lymph nodes, notably the popliteal, in rabbits with a latent infection. It would seem that other factors than serologic antibody reactions influence the causation of latency, or also smaller quantities of reagin than may be detected by the usual Wassermann test are sufficient to hold in check the remaining foci of spirochetes. These findings are not inconsistent with the clinical observations in man since latent and even occasionally active syphilis sometimes occurs in the presence of a negative Wassermann test. It should be emphasized that other observations in correlation with the Wassermann test must be kept in mind in the examination and treatment of syphilitic infections.

SUMMARY

Observations of the Wassermann reactions in experimental syphilis of rabbits indicate that the serologic response is not identical in every instance. There is also a vacillation in the reaction to some extent. These results in rabbits suggest that if repeated Wassermann tests were made in cases of suspected latent syphilis, many patients might be found to be syphilitic whose syphilis is now overlooked.

[1] Jour. Exper. Med., 1922, 35, p. 39.

A MODIFIED METHYL ORANGE INDICATOR

SUITABLE FOR USE WITH ARTIFICIAL LIGHT

One Plate

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Methyl orange, or helianthin, as it is sometimes called is an exceedingly useful indicator because it is not affected by such a weak acid as carbonic acid. It is suitable for titrating inorganic acids, alkalis, alkali carbonates or bicarbonates, but it cannot be employed in titrating organic acids or alcoholic or boiling solutions. This indicator gives, as is well known, a yellow color with alkalis and a rose color with acids.

The color change from the acid or alkaline side to the end point is a difficult one to determine especially for one who has not had considerable experience with this indicator. This end point is rendered even more difficult, in fact, almost impossible to recognize if titrations are performed by artificial light.

Luther [1] added indigo carmine to methyl orange in titrating yellow solutions. The color changes from an alkaline to an acid solution are: yellow, green, colorless (gray), violet. He concludes that, "This succession of colors lessens the danger of passing the end point in rapid titrations." Hickman and Linstead [2] employed xylene cyanole instead of indigo carmine to hasten the ease of arriving at the end point.

As has been said above, it is practically useless to attempt to perform titrations with artificial light. It was found that the determination of the end point either with natural or artificial light is a simple matter if methyl orange is combined with acid green (sodium salt of diethyl-dibenzyl-diamido-triphenyl-carbinol-trisulphonic acid) and acid blue (sodium salt of m-oxy-diethyl-diamido-phenyl-ditolyl-carbinol-disulphonic acid). The scale from the alkaline to the acid side is as follows: bright green, pale green, gray (end point, P_H 3.8), pale violet, violet. The color change is a sensitive one, and the indicator can be easily employed at night, even by the inexperienced worker.

Received for publication, Nov. 30, 1925.

[1] Chem.-Ztg., 1907, 31, p. 1172.
[2] J. Chem. Soc., London, 1922, 121, p. 2502.

Acid green and acid blue are indicators which do not alter their property either in a strongly acid or a strongly alkaline solution. Their function is merely to modify the color of the methyl orange. If the methyl orange is yellow the combination of colors will give a grass green, and if the methyl orange is rose the combination will give a violet. Acid green in solution assumes a blue green color while acid blue assumes a pale violet color.

The indicator is prepared as follows:

Methyl orange	0.1125 Gm.
Acid green	0.1500 Gm.
Acid blue	0.0375 Gm.
Total	0.3000 Gm.

The dyes are dissolved in 100 cc. of 50% alcohol and the resulting solution filtered. Three or four drops will be found sufficient for 100 cc. of solution to be titrated.

The indicator may also be employed for the colorimetric determination of hydrogen ions. The range of its usefulness will be found to lie between P_H 2.6 and 4.2, as can be seen from the accompanying chart. The color change from green to purple is one to be desired as the comparison of colors can be easily performed with artificial light just as in the titrimetric method.

CONCLUSIONS

A modified methyl orange indicator is described consisting of methyl orange, acid green and acid blue which may be used with artificial as well as with natural light for titrations and for the colorimetric determination of hydrogen ions having a range between P_H 2.6-4.2.

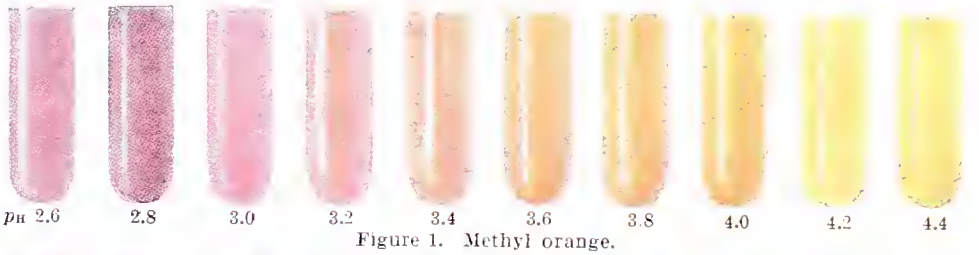

Figure 1. Methyl orange.

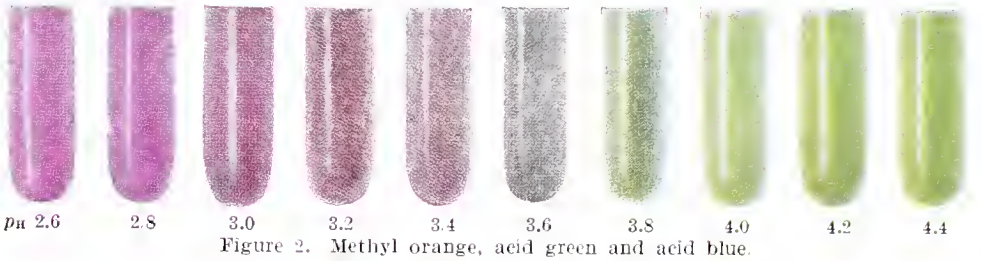

Figure 2. Methyl orange, acid green and acid blue.

HUMAN INTESTINAL CARRIERS OF TETANUS SPORES IN CALIFORNIA

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It has been stated repeatedly in the literature that man may carry tetanus bacilli in his digestive tract, the statement apparently being based on the work of Pizzini [1] early in 1898. Pizzini injected suspensions of feces from 100 individuals into guinea-pigs, and in 5 instances the animals developed tetanus symptoms. The general conclusion drawn from this work was that only those who are in intimate contact with horses are likely to be carriers of this organism. No records of other studies on this subject are found in the literature until 1919 when Tulloch,[2] during his extensive work on tetanus, became interested in the digestive tract as a possible source of tetanus infection. He studied the feces of 21 men returned from overseas service and in 7, or 33%, isolated tetanus bacilli. From the feces of 33 civilians in England he obtained this organism in 5 instances, or 16%. Ten Broeck and Bauer [3] in 1921 examined the stools of 78 Chinese hospital patients in Peking and found 34.7% harboring tetanus spores in their feces. In 1924 [4] the same workers undertook a more extensive survey of tetanus carriers in various parts of China, and of 539 human fecal specimens investigated, they obtained this organism in 153 instances, or 26.5%. Fildes [5] examined the feces of 200 persons in England and succedeed in isolating tetanus bacilli in only two cases. The findings of this worker are rather surprising, since they differ considerably from those of Tulloch, who only 5 years before isolated tetanus bacilli in 16% of cases in the same country. The difference in the results may be due to the method employed by Fildes, which is so radically different from all those used by other workers. Buzello and Rahmel [6] examined the feces of 50 individuals in Germany, and demonstrated the presence of tetanus

Received for publication, Dec. 16, 1925.

[1] Centralbl. f. Bakteriol., I, O., 1898, 24, p. 890.

[2] J. Hyg., 1919-20, 18, p. 103.

[3] J. Exper. Med., 1922, 36, p. 261.

[4] Paper presented at the Congress of the Far Eastern Association of Tropical Medicine in Tokyo, October, 19[illegible].

[5] Brit. J. Exper. Path., 1925, 6, p. 62.

[6] Arch. f. klin. Chir., 1924, 80, p. 660.

bacilli in 20 instances, or 40%. Van der Reis,[7] while studying human intestinal flora in general, in one of his patients with secondary anemia found a practically pure culture of tetanus bacilli in the large intestines. Kahn,[8] investigating the anaerobic intestinal flora of 72 persons in New York City, found none containing tetanus spores.

The object of Kahn's experiment was primarily to estimate the number of spore-bearing anaerobes present in the feces of persons with various pathologic conditions, such as intestinal toxemia, chronic diarrhea, skin eruptions, arthritis, pernicious anemia, etc. For this purpose he diluted his material considerably in order to obtain well isolated colonies in shake cultures. There is a possibility that tetanus spores were not as numerous as the other spore-bearing anaerobes commonly present in the feces, and therefore escaped detection.

The records in the files of the State Board of Health show that there have been in the State of California, during the period from Jan. 1, 1922, to Dec. 1, 1925, 245 cases of tetanus, with 164 deaths, the mortality being 67%. According to the histories of these cases, there is a large number such as tetanus neonatorum (20 cases), tetanus following abortion, etc., in which the infection probably has been of fecal origin.

So far as we know, no studies have been made in the United States on the distribution of human tetanus carriers in general, and since this disease seems to be more prevalent in California than in the central and eastern States of the Union, we thought it worth while to undertake a survey of human tetanus carriers in this state.

Most of the material for this study was received from Dr. C. A. Kofoid, who was carrying on an extensive parasitologic survey in California, and receiving fecal specimens through the health officers from different parts of the State. Dr. Kofoid kindly sent these specimens to us after they had been examined for the presence of intestinal parasites. Smaller proportions of our material were received directly from various hospitals in San Francisco and Los Angeles.

TECHNICAL PROCEDURE

A clump of fecal matter about the size of a bean, or in case of liquid stool, 1 or 2 cc., was transferred with a sterile glass rod or pipet directly into a tube of beef heart medium, which was then heated at 70-75 C. for 20 minutes, cooled rapidly in cold water and incubated

[7] Ergebn. d. inn. Med. u. Kinderh., 1925, 27, p. 77.
[8] J. Infect. Dis., 1924, 35, p. 423.

for 5-7 days. Two smears were made from each culture, usually on the fourth and sixth day of incubation, and examined under the microscope for the presence of tetanus-like organisms. After some experience it is relatively easy to recognize tetanus bacilli among other round or oval terminal spore-bearing organisms which are commonly present in these cultures. Those which were found positive for tetanus-like organisms were transferred into freshly prepared Tulloch's [9] exhaust medium and incubated for 5 days. This medium has certain selective properties for tetanus bacilli and is valuable for the isolation of this organism from any source. After 5 days of incubation, tetanus bacilli usualy overgrow all other organisms in this medium, and isolation presents no difficulties. A loopful of the culture in Tulloch's medium is suspended in a tube of about 10 cc. of salt solution, and, after shaking vigorously, one loopful of this suspension is transferred into a deep tube of clear melted 2% liver agar at 70-80 C., which is then cooled rapidly and incubated for 18-24 hours. On the next day, each tube thus prepared will contain approximately 2-10 well isolated colonies. As tetanus colonies are rather characteristic and predominating in such cultures, they are easily recognized among the others. The bottom of the tube is carefully heated over a flame to force out the column of the agar into a sterile Petri dish, where it is cut into rather thin slices with a red hot safety razor blade. Typical colonies are then fished from the cut surface with a platinum rod and transferred into beef heart medium. For fishing the colonies fine capillary pipets have previously been used in this laboratory for botulism work, but we found platinum wire as efficient and much more practical than pipets. By using this technic we succeded in isolating tetanus bacilli without difficulty from the first shake culture; and in a few instances these were either heavily seeded or contained no colonies, then the procedure had to be repeated until pure cultures were obtained. We did not fail, however, to isolate tetanus bacilli from fecal cultures in any case which had on microscopic examination been diagnosed as positive for this organism.

All strains isolated were tested for toxicity, and their serologic type determined. For the toxin test 5-7 day old broth cultures were used, which were centrifugalized at a high speed for 30 minutes to remove the bacteria and spores from the supernatant fluid. Three white mice were inoculated with each culture: two received 0.01 and 0.001 cc., respectively, of the clear supernatant fluid, while the third

[9] Proc. Roy. Soc., B., 1919, 40, p. 529.

mouse received 0.1 cc. of the same culture mixed with 3 units of commercial tetanus antitoxin. The tetanus type cultures employed for the production of type immune serums were the same strains which Ten Broeck and Bauer used in their tetanus studies. For immunization, 24-hour broth cultures were used which had been washed with salt solution and heated to 56 C. for one hour to kill the organisms. Rabbits were given two intravenous injections of heated tetanus bacilli with an interval of 5 days, the dose being approximately 2 billion for the first injection and 3 billion organisms for the second injection. The animals were bled on the seventh day after the last injection, and in case the agglutinating titer was not sufficiently high, another injection of 3 billion organisms was given. In most cases 2 injections were sufficient to obtain a titer of 1 : 6000-1 : 12000. As antigens for the agglutination test, 24-hour cultures grown in peptic digest broth were used. Straight broth cultures were used, since it was found that suspensions of washed tetanus bacilli, especially of type 3, had a tendency to spontaneous clumping. Of 129 strains isolated in this experiment, cross agglutination was observed only with 3 cultures and their serologic type was determined by an absorption test.

EXPERIMENTAL RESULTS

Data on Tetanus Carriers.—Residence: Dr. C. A. Kofoid, for the purpose of his studies, received most of the fecal specimens from various counties in the vicinity of San Francisco, and since we have largely used his material for our experiment, a relatively small number of specimens have been examined from persons of the inland, particularly of the northern part of California. For convenience in analyzing the results, the state has been divided into valleys and bay regions, which are the principal centers of human habitation. A total of 487 fecal specimens were examined, and in 120 instances tetanus bacilli have been isolated, the incidence of carriers being 24.6%. In addition to these, 43 stools were received from 19 other states of the Union and in 9 cases, or 26.6%, this organism was obtained. As seen in table 1, the incidence of carriers varies from 6.6 to 39.1% in different localities without any apparent geographic or climatic relationship. The incidence seems rather low on the southern sea coast, while relatively high in the southern inland, especially in the San Joaquin Valley (Kern County). Unfortunately, only 3 specimens were received from Hum-

boldt County and naturally no conclusion can be drawn from such a small number.

TABLE 1

NUMBER OF TETANUS CARRIERS IN CALIFORNIA AND OTHER STATES

Localities in California Where Specimens for Examination Were Collected	Number Positive	Number Negative	Total Number Examined	Percentage of Carriers Found
San Francisco Bay region	65	180	245	26.5
San Joaquin Valley	18	28	46	39.1
Santa Clara Valley	12	38	50	24.0
Los Angeles County	14	69	83	16.8
Napa Valley	3	16	19	15.7
San Diego	1	14	15	6.6
Sacramento Valley	6	20	26	20.7
Humboldt County	1	2	3	33.3
Total for California	120	367	487	24.6
Other states of the Union	9	34	43	26.6
Grand total	129	401	530	24.1

Birthplace: Most of the persons examined were not natives of California. It was first supposed that if a person is a carrier for a considerable length of time, and had been infected in his native place before settling in this state, an analysis of the birthplaces might give some information as to whether certain states or countries are more heavily infected than others. But, as indicated in table 2, the geographic

TABLE 2

NUMBER OF TETANUS CARRIERS IN DIFFERENT STATES AND COUNTRIES

Birthplace	Number Positive	Number Negative	Total Number Examined	Percentage of Carriers
California	37	112	149	24.8
Other states of the Union and Canada	48	152	200	24.0
Europe	10	31	41	24.3
Mexico, Philippine Islands, India and Far East	4	24	28	14.0
No data received	30	82	112	26.7
Total	129	401	530	24.1

distribution of carriers, according to their birthplaces, is more or less equal in California to those of other states of the Union, Canada and Europe, with the exception of a few persons, natives of tropical or subtropical countries.

Sex: The sex distribution, shown in table 3, is practically equal among the carriers as well as noncarriers.

Age: The average age of the persons examined was between 35 and 40; the youngest being 1½ years and the oldest 85 years of age. Among those that were found positive for tetanus spores, the youngest was 3½ years and the oldest 80 years, the age of the majority being between 30 and 40.

TABLE 3

SEX DISTRIBUTION OF TETANUS CARRIERS

Location Where Specimens Were Collected	Males		Females		Total
	Positive	Negative	Positive	Negative	
California	62	196	58	171	487
Other states	6	22	3	12	43
Total	68	218	61	183	530

Occupation: It appears from the results of our experiment that occupation plays practically no role in the distribution of tetanus carriers. Of 129 patients with positive cases, only 9 were farmers and ranchers, who may have been in more or less close contact with horses, whereas the remaining 120 were students, clerks, typists, mechanics, stenographers, cooks, salesmen, housewives, etc., who had practically nothing to do with horses or other domestic animals.

TABLE 4

SEROLOGIC TYPES OF TETANUS BACILLI ISOLATED FROM STOOLS IN CALIFORNIA

Location in California Where Stool Specimens Were Collected	Type 1	Type 2	Type 3	Type 4	Type 5	Inagglutinable Strains	Total
San Francisco Bay region	44	5	11	1	2	2	65
San Joaquin Valley	13	..	3	1	..	1	18
Santa Clara Valley	5	1	5	..	1	..	12
Sacramento Valley	3	2	..	..	1	..	6
Los Angeles County	10	1	1	..	2	..	14
San Diego	..	..	..	1	..	..	1
Napa Valley	2	..	1	..	..	..	3
Humboldt County	1	..	..	..	..	..	1
Total for California	78	9	21	3	6	3	120
Other states of the Union	4	..	5	..	..	..	9
Grand total	84	9	26	3	6	3	129

TYPES OF TETANUS BACILLI ISOLATED

As to the distribution of different serologic types of tetanus bacilli, it is interesting to note from table 4 that type 1 was found predominating in all localities, with the exception of Santa Clara Valley, in which types 1 and 3 were found in equal number. Among the others,

type 3 was more or less prevalent, while types, 2, 4 and 5 were relatively rare, and type 6 was not encountered.

We also found 3 strains which were morphologically and culturally true tetanus bacilli, and which produced toxin that was neutralized by commercial tetanus antitoxin, but they were not agglutinated in any of our 6 type serums. Rabbits were immunized with each of the 3 strains and their serum tested with the culture used for immunization, as well as with the other 2 strains. It was found that each serum agglutinated all 3 strains to the titer limit, indicating that they belong to one serologic group.[10]

Comparing our findings with those of other workers in different countries as seen in table 5, it is interesting to note that according to

TABLE 5

TYPES OF TETANUS BACILLI FOUND IN DIFFERENT COUNTRIES

Results Obtained by	Number of Tetanus Cultures Agglutinating in Serum of Type							Total Number of Strains Studied
	1	2	3	4	5	6	7	
Tulloch on cultures isolated from war wounds in France	60	25	35	5	1	0	0	127
Tulloch on fecal cultures in England	9	2	1	0	0	0	0	12
Fildes on cultures from feces of mau and horses, soil, wounds og cases of tetanus, etc., in England	13	6	14	3	39	0	0	75
Bauer, Tang, Yü and Ten Broeck on cultures from the feces of man and domestic animals in China	21	17	52	1	155	10	5	261
Our cultures from human feces in California	78	9	21	3	6	0	?	117*

* The 3 strains which were not agglutinated in any of the 6 type serums are not included in this table.

Tulloch's results type 1 is prevalent also in France and England. Fildes' figures from England differ greatly from those of Tulloch, and agree more or less with the findings in China. It must be remembered, however, that Tulloch tested only 12 fecal cultures; of these, 7 were isolated from feces of men recently returned from overseas service in France where type 1 seems to be predominating, and only 5 were obtained from civilians in England. Type 5, which Tulloch found only once in France and we obtained six times in California, seems to be highly prevalent in China and according to Fildes also in England.

[10] In this laboratory there were 20 stock cultures collected by Heller (J. Infect. Dis., 1922, 30, p. 18) from different serum institutions and laboratories in the United States. These strains have been isolated from various sources other than feces in this country and are used in some institutions for the production of commercial tetanus antitoxin. They were tested with type serums and 18 were found to be type 1, while only 2 were agglutinated in type 3 serum.

TOXICITY

All strains isolated in this series were found to form toxin which when injected into white mice produced typical tetanus symptoms, and was neutralized by commercial tetanus antitoxin. Most of our cultures killed mice in doses of 0.01 and 0.001 cc. within 2-4 days, and only 2 strains were encountered of which 0.1 cc. was necessary to produce generalized tetanus and death in the same time. It is surprising that Fildes [11] found practically half of his cultures atoxic; even some of the strains which produced toxin at the beginning of his experiments became atoxic by the time his studies were completed. On the other hand, Ten Broeck and Bauer isolated nearly 300 strains of tetanus bacilli in China and did not find a single culture that failed to produce toxin; likewise Tulloch in his papers does not mention having encountered any atoxic strains.

DISCUSSION

The results of our investigation show that one fourth of the population of California carries tetanus bacilli in the digestive tract. Since this is the first study of this kind in the United States which covers only a small part of the territory, it is probable that different results may be obtained in other parts of the country. We also admit the possibility that by our technic some of the positive cases may have escaped detection, and that our figures are lower than the actual number of carriers. On the other hand, Ten Broeck and Bauer [12] controlled a series of their fecal examinations by blood tests. They could never find antitoxin in the blood when tetanus spores could not be found in the feces; and since we have used their technic in our studies, it seems unlikely that many positive cases have been missed.

Judging from the fact that a relatively small amount of fecal matter has been used for examination and that antitoxin has been found in the blood of tetanus carriers by other workers (Ten Broeck and Bauer,[12] van der Reis [7]), it is evident that this organism actually multiplies and sporulates in the digestive tract of man. Just how much human excreta actually contaminate soil with tetanus spores in comparison with animals is difficult to estimate, since human feces in civilized countries, and especially in big cities, are better disposed of from a sanitary point of view than those of domestic animals also known to be carriers of this organism. The fact, however, that approximately 1 gm.

[11] Brit. J. Exper. Path., 1925, 6, p. 91.
[12] J. Exper. Med., 1923, 37, p. 479.

of fecal matter of every fourth individual in this State contains tetanus spores, leads to the conclusion that an enormous amount of infectious material capable of producing fatal disease under favorable circumstances is discharged daily by these carriers.

We would suggest that studies of this kind be carried out in other parts of the United States, and that special emphasis be laid on the determination of various types of the tetanus bacilli involved. This is especially important as Ten Broeck and Bauer [13] have pointed out in their latest publication on tetanus type immunity that tetanus carrier guinea-pigs are immune to the type of bacilli they carry. However, these can be easily infected with another type, and they die in the same time as noncarriers, regardless of the amount of antitoxin present in the blood of such carriers. It is probable that a human carrier is likewise more or less immune to the type of bacilli he carries; he is, nevertheless, dangerous to those who are not carriers or who carry other types.

The question arises as to whether an individual once infected remains a carrier for a considerable length of time or for a relatively short period. The fact alone that specific antibodies to this organism, such as antitoxin and agglutinins, are found in the blood of the carriers, favors the hypothesis that a carrier must harbor these organisms over an extended period. This view is supported by the findings of Ten Broeck and Bauer,[3] who could demonstrate an enormous number of tetanus spores in the feces of a patient that had been on practically sterile food in the hospital for 105 days.

It is evident that in such a case the digestive tract must offer optimum conditions for the organism to grow, and one would naturally think that if conditions are generally favorable, all individuals should eventually become tetanus carriers. We know, however, that this is not the case. The larger proportion of the population are not carriers, although tetanus spores are unquestionably frequently ingested with food in this proportion. This would probably indicate that the tetanus bacillus is not a common intestinal inhabitant, and that certain factors must be involved which permit the organisms to establish themselves only in a limited number of persons. One of these factors is probably the intestinal flora, which may be symbiotic to the tetanus bacilli in one person and antagonistic in another. We have noticed that the microscopic picture of the fecal culture of a carrier differs from that of a non-

[13] Proc. Soc. Exper. Biol. & Med., 1925, 12, p. 562.

carrier in that the tetanus bacillus is always accompanied by certain oval or round terminal spore-bearing anaerobes, and we have never seen tetanus bacilli present in the feces when these organisms are not to be found. In our opinion, there seems to exist a mutual relationship between these organisms and the tetanus bacillus, at least when they grow in the intestinal tract of man, although we have not attempted to study this problem.

It has been the belief that the small intestines offer the best anaerobic conditions for bacteria to grow, but van der Reis,[7] who is probably the best authority on the intestinal flora, and who has made exhaustive studies along this line, has shown conclusively that there are no anaerobes in the small intestines. The common intestinal spore-bearing anaerobes, tetanus bacillus included, are inhabitants primarily of the cecum. They are less numerous in the colon and only occasionally found in the lower part of the ileum. Taking into consideration that in cultures tetanus bacillus usually begins to form spores after 48 hours, and whereas spores are abundantly present in the feces of a carrier it would seem that this organism belongs to the parasites of the intestinal wall rather than to the chyme saprophytes, otherwise the majority of them would be evacuated mechanically by the fecal masses in vegetative form before sporulation. If this hypothesis is true, there must exist a certain individual disposition in the intestinal mucosa of the carrier which will tolerate the presence of this organism. It may be that this disposition is only temporary, and that after a certain length of time either general or local immunity is developed in the host, and the parasite disposed from the intestinal tract. We know from the experiment of Noble,[14] who examined the feces of a horse—a natural carrier—over a period of 7 months, and during the first 4 months found tetanus spores on every examination. The organisms then suddenly disappeared, and on subsequent repeated examinations during 3 months the findings were continuously negative. Likewise agglutinins have been found by Ten Broeck and Bauer [12] in the blood of individuals in whom tetanus spores could not be detected in the feces. This would probably indicate that such a person had been intimately associated with this organism some time in the past.

These are exceedingly interesting points which have not yet been investigated.

[11] J. Infect. Dis., 1915, 16, p. 132.

SUMMARY

Four hundred and eighty-seven specimens from California have been examined, and 24.6% were found positive for tetanus spores.

In addition to these, 43 specimens were examined from 19 other states of the Union, and in 9 instances tetanus bacilli have been obtained. Although no conclusion can be drawn from such a small number, it nevertheless indicates that tetanus carriers are also to be found in other parts of the country.

Sex, age and occupation apparently play no part in the distribution of tetanus carriers.

No atoxic strains have been found in this series.

Of the 120 tetanus bacilli isolated from human feces in California, 63.3% were type 1; 7.5%, type 2; 17.5%, type 3; 2.5%, type 4, and 5%, type 5. Type 6 has not been found.

In addition to these, three strains of tetanus bacilli have been found which were not agglutinated with any of six type serums tested. Unfortunately type 7 serum has not been available and therefore it is not determined whether these strains are type 7 or belong to a new serologic group heretofore not described.

THE CHANGES IN THE BACTERIAL CONTENT OF STORED NORMAL AND TYPHOID FECES *

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The bacterial changes that occur in the contents of privy vaults and earth closets seem never to have been fully studied, although the bacteriology of sewage has been the subject of much investigation.

THE BACTERIA OF FRESH NORMAL FECES

Number.—No attempt has been made in this study to determine the total number of viable bacteria in feces. The difficulties of such an undertaking are recognized by most workers and have been clearly set forth by Harris.[1] For purposes of comparison the ordinary standard agar count at 37 C. is probably as useful as more elaborate methods and combinations of methods. Some of the earlier observations on the number of cultivable bacteria in stools show considerable variation. Sucksdorff,[2] using gelatin as a culture medium, found a range of 2,300,000 to 25,000 per mg. and deemed 381,000 an average. Gilbert and Dominici [3] recorded an average of 67,000 per mg., Casciani,[4] 16,000, and Matzuschita,[5] 18,000,000. Unfortunately, the methods used in obtaining these counts were not uniform. Later results obtained by methods which are fairly comparable give more uniform results. MacNeal, Latzer and Kerr,[6] using litmus lactose agar in the examination of 202 stools from 12 subjects, record a maximum of 1,526,140 bacteria per mg. of feces, a minimum of 1,360 and a mean of 116,239. Houston,[7] who used an agar medium for examining 17 stools, found 4,140,000 the maximum, 2,800 the minimum and 373,000 the average (per mg. of feces). Harris [1] gives a range from 950,000 maximum to 10,000 minimum, using standard agar medium and 30 stools from 6 subjects.

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* This study was undertaken as part of the work of the Board on Excreta Disposal of the U. S. Public Health Service. Mrs. O. T. McArthur and Miss Dorothy Ashland have assisted.

[1] J. Am. M. Assn., 1912, 59, p. 1344.

[2] Arch. f. Hyg., 1886, 4, p. 355.

[3] Compt. rend. Soc. de biol., 1894, pp. 117, 277.

[4] Deutsch. med. Wchnschr., 1897, 23, p. 297.

[5] Inaug. Diss., Halle, 1902, cited by Harris.[1]

[6] J. Infect. Dis., 1909, 6, pp. 123, 571.

[7] Local Gov't. Bd., Report of Med. Officer, 1902, 7, p. 511.

In the present study we also used the standard agar and obtained from 41 stools of 24 subjects the following figures: maximum 530,000, minimum 790, average 77,220.

The B. coli Group.—The majority of the bacteria in fresh feces which are capable of developing colonies on the usual culture media have been regarded by nearly all workers as belonging to the B. coli group. Houston [7] found B. coli in numbers ranging from 100,000 to 10,000,000 per mg. and believed an average of between 100,000 and 1,000,000 per mg. a fair estimate. MacNeal, Latzer and Kerr [8] note that "The number of colonies on . . . aerobic litmus lactose gelatin, generally in close agreement, represents approximately the quantity of cultivable B. coli in the feces. Other bacteria occurred in small numbers and not constantly." This would give numbers considerably less than those reported by Houston [7] who found that 251 out of 257 colonies isolated from 12 stools produced acid and gas in lactose peptone medium and concluded that atypical coli-like microbes were far less common than the typical in the normal stools of healthy persons.

Methods.—The observations here recorded were made upon the fresh stools of healthy normal individuals (24 subjects, 41 stools). The methods of mixing, diluting, etc., were essentially those employed by MacNeal, Latzer and Kerr [6] and by Harris.[1] A small sterilized glass spoon was used for mixing one gram of the feces with water in a watch glass, and great care was taken to secure a fine and uniform suspension. The first dilution flask (1 gm. of feces to 100 cc. of water) was shaken vigorously for 10 to 15 minutes to complete the mixing. One cc. of each of the dilutions selected was used in plating. The most suitable dilutions in routine were found to be 1 : 10,000, 1 : 100,000 and 1 : 10,000,000.

For the colony count, standard agar was used and the plates were incubated for 48 hours. For the B. coli enumeration the medium of Ayers and Rupp [9] was found very satisfactory (48 hours' incubation).

Comparative tests of four mediums made with suspensions of pure cultures of B. coli showed, by comparison with the standard agar medium, that there is little, if any, inhibition of strains of the coli-aerogenes group by the Endo medium and by the Ayers and Rupp medium, but that the earlier Ayers and Johnson medium [10] exerts con-

[8] J. Infect. Dis., 1909, 6, p. 577.
[9] J. Bacteriol., 1918, 3, p. 433.
[10] Bull. 303, U. S. Dept. of Agric., 1915.

siderable inhibitory effect. This inhibiting action was in fact noted by Ayers and Johnson in their original paper.[11]

Although there is not much, if any, difference between the suitability of the Endo medium and the Ayers and Rupp medium for the growth of B. coli, the latter medium has been found to give a sharper and more uniform differentiation of the colonies on plates of fecal suspensions, and hence, after a series of comparative tests, has been used for the B. coli enumerations here recorded.

A special study by Miss Ashland demonstrated that the Ayers and Rupp medium is highly reliable in developing colonies of the coli-aerogenes group sufficiently characteristic to be distinguishable from the colonies of other bacteria and that a count of these colonies gives a close approximation to the number of organisms of this group in the feces plated.

TABLE 1

COLONY COUNT (AGAR 37 C.) AND B. COLI ENUMERATION (AYERS AND RUPP) PER MG. IN DIFFERENT PORTIONS OF THE SAME STOOL

Portion	Stool 1		Stool 2		Stool 3	
	Colony Count	B. coli	Colony Count	B. coli	Colony Count	B. coli
1	130,600	112,200	52,000	45,000	109,500	59,500
2	59,000	70,400	79,000	75,000	61,500	8,500
3	68,800	53,300	141,000	186,000	75,500	24,000

Ratio of B. coli Count to Total Colony Count.—It seemed desirable to determine the extent of variation in bacterial content in different parts of one and the same stool.

The results recorded in table 1 are typical of a large series. The ratio of the B. coli count to the total colony count on agar at 37 C. is not constant, but varies in different stools and to a lesser degree in different portions of the same stool. In 24 apparently healthy subjects the ratio of the B. coli count to the total colony count ranged from 0.3% to 100%; it usually was about 70-80%.

THE BACTERIA OF STORED NORMAL FECES

I have not been able to find in the literature any detailed observations on the changes that occur in feces stored under such conditions as are likely to obtain in closed privy vaults, earth closets and the like.

[11] Bull. 303, U. S. Dept. of Agric., 1915, p. 20.

In carrying out the observations here set down, fecal masses have been kept in the dark in galvanized iron cans with rather tightly fitting covers to prevent rapid evaporation.

A thin dry crust usually forms on the surface; the samples for the bacterial examination were always taken from the inner moist portion. Determination of the moisture content showed that the amount of water in the fresh samples tested ranged from about 77 to 85% and changed but little in the course of several weeks. Three different samples tested after 11 weeks showed respectively a moisture content of 80, 80.3 and 81%. Under the conditions in which these experiments were conducted there seems no reason to attribute the changes in the bacterial content of the feces to drying.

Numerical Changes on Storage. Total Colony Count.—The first change observed in normal stools stored at room temperature was a marked increase in the colony count during the first few days of storage. The following examples are typical of a larger number:

TABLE 2

CHANGES IN COLONY COUNT IN FIRST DAYS OF STORAGE

	Subject E	Subject C	Subject F
Initial number of colonies per mg.	50,000	18,000	210,000
After 24 hours storage	1,120,000	19,400	.....
After 3 days storage	.....	104,500	10,000,000

In some instances the numbers of cultivable bacteria were higher throughout the first 2 weeks of storage than they were initially, but as a rule a decline in numbers made its appearance before the 15th day of storage. This decline was in some instances fairly rapid and uniform; in others the numbers remained for a long time higher than the initial number, as in table 3.

TABLE 3

CHANGES IN COLONY COUNT IN FECES STORED AT ROOM TEMPERATURE, TAKING INITIAL NUMBER AS 100

Storage Period in Days	Count	Storage Period in Days	Count
5	2,928	16	533
7	2,714	18	445
9	2,023	21	378
11	1,057	28	800
14	695		

In one instance the colony count after 84 days was more than 15 times the original number per mg. (535,000 : 34,500).

Observations of this character have been continued for as long as 21 weeks with the result that during the later weeks of storage the numbers, while usually lower than the initial numbers, are nevertheless actually high, showing that feces stored for from 4 to 5 months are very far from having lost their availability as a medium for bacterial existence. Although, as pointed out, there is some loss in the moisture content of the feces in storage, it is evident that this is not sufficient to cause any noteworthy reduction in the number of bacteria capable of growing on transplantation to nutrient agar.

In summing up the changes in the total colony count of bacteria in feces stored at room temperature (13 experiments) the following statements seem justified: (1) In all instances the number of bacteria increases during the first few days of storage. (2) A decline in numbers usually sets in within the first two weeks of storage, but this is not invariable. (3) The actual number after two or three months of storage is often less than the initial number in the fresh stool, but this is by no means always the case, and in more than one-third of the experiments the colony count was higher at the end of the storage period than at the beginning. (4) Great variation is observed in the actual numbers and in the proportion and rate of change in different stools. This is doubtless to be expected since among other factors the amount of nutrient material in fresh feces which is available for bacterial growth must vary greatly.

Effects of Temperature. Storage at 37 C.—Six lots of feces were stored in the incubator at 37 C. It was impossible to avoid altogether the effects of drying and on this account the experiments were usually discontinued after about three weeks. In two instances, however, on the 23d and 24th days, respectively, the colony count showed several hundred times as many colonies as the original fresh sample. The general course of events was strikingly similar to that observed at room temperature. There was first a multiplication, and this was followed by a more or less irregular decline which in 3 of the 6 experiments brought the actual numbers lower than the initial numbers (in about 3 weeks) and in the other 3 left them considerably higher. The following experiments are typical:

TABLE 4

CHANGES IN COLONY COUNT IN FECES STORED AT 37 C., TAKING INITIAL NUMBER AS 100

Storage Period	Counts	
	Exper. 22	Exper. 29
3 hours	280	178
6 hours	1,066	153
24 hours	28,571	26,538
48 hours	22,417	17,884
3 days	4,285	5,423
6 days	1,099	1,192
8 days	2,492	635
9 days	5,494	384
10 days	660	...
12 days	714	...
14 days	769	923 (13 days)
17 days	2,153	...
20 days	80	384 (19 days)

Storage at 10 C.—Eight lots of feces were stored in the refrigerator at approximately 10 C. The bacterial changes as shown by the colony count were essentially the same as those observed at 37 and at room temperature. There was first a multiplication leading to numbers considerably in excess of the numbers in fresh stools, and then an irregular, generally very gradual decline. The effect of the relatively low temperature was shown by the slow initial multiplication as compared with that taking place at 37, and by the long persistence of comparatively high numbers. In one instance the colony count after 8 months was somewhat higher than the initial count and in another, after 23 weeks, the count compared with the initial count as 573 : 100. The following experiment fairly represents the general conditions observed:

TABLE 5

CHANGES IN COLONY COUNT IN FECES STORED AT 10 C., TAKING INITIAL NUMBER AS 100

Storage Period in Weeks	Count	Storage Period in Weeks	Count
1	964	10	700
2	418	12	564
3	327	16	80
4	327	20	600
5	754	22	855
6	225		

The majority of the experiments showed, as in the instance cited (table 5), a less extensive multiplication at 10 C. than occurred at the higher temperatures; but in one of the experiments a considerable bacterial increase was observed, which at its highest point compared with the initial number as 14,500 : 100 (10 days) and 17,600 : 100 (20 days).

Storage at —11 C.—Three lots of stools were frozen and held at —11 C. for periods ranging from 15 to 18 weeks. No initial multiplication was noticed in these cases. The lack of substantial reduction in the bacterial content of these frozen stools was striking.

TABLE 6

CHANGES IN COLONY COUNT PER MG. IN FECES STORED AT —11 C.

After Storage Period	Count	After Storage Period	Count
Initial	106,000	51 days	30,500
1 day	96,000	8 weeks	35,000
5 days	44,500	9 weeks	16,000
8 days	143,640	10 weeks	40,000
12 days	107,000	11 weeks	25,000
15 days	47,500	12 weeks	15,000
20 days	275,000	13 weeks	5,570
23 days	24,500	14 weeks	56,000
27 days	36,000	15 weeks	13,500
30 days	40,000	16 weeks	9,500
35 days	35,250	17 weeks	3,000
40 days	40,000	18 weeks	15,000

The effects of temperature on the bacterial changes in stored feces may be summed up by saying that in general the high temperatures were accompanied by a more rapid initial multiplication, and that the actual bacterial numbers averaged somewhat higher in the stools kept at room temperature and at 37 C. than in those kept at 10 C. It is noteworthy that an initial increase occurred throughout a rather wide temperature range. In frozen stools kept at —11 C., the reduction in bacterial count was not so great as might have been anticipated.

TABLE 7

CHANGES IN COLONY COUNT IN FECES STORED UNDER GARDEN SOIL AT ROOM TEMPERATURE, TAKING INITIAL NUMBER AS 100

Storage Period in Days	Count	Storage Period in Days	Count
4	8,756	72	396
10	547	86	74
17	597	98	78
24	413	112	2
33	330	126	103
56	317		

Storage under Garden Soil.—The bacterial changes in fecal masses under a rather heavy covering of garden soil at room temperature have been studied in the same way as above. Table 7 is typical of the series (7 experiments). The well-known phenomenon of "absorption" of the feces by the soil made it impossible in the later stages of the experiments to identify the fecal masses as such by ordinary naked-eye inspec-

tion. Their original position was, however, definitely marked. So far as ordinary examination could determine, the absorption was usually complete in from 10 to 12 weeks.

The changes in the bacterial content of the feces stored under garden soil were essentially the same as those in feces stored without covering. An initial multiplication followed by an irregular decline was observed in all cases. Total numbers often remained high. The rather great fluctuations in the colony count after absorption is complete may be due to the natural irregularities of sampling. The position occupied originally by the fecal mass is, however, recognized for many weeks by the high colony count. The colony count of the garden soil used in these experiments averaged about 300 per mg. In the experiment that was carried out for the longest period the initial count of the fresh feces was 130,000 per mg. The feces were to all appearances "entirely absorbed" by the soil at the end of 12 weeks, but counts made after 26, 28, 30 and 32 weeks gave respectively 17,000, 11,000, 45,000 and 20,500, numbers much higher than the 300 in the soil without fecal addition. In another experiment the initial fecal count was 38,000; absorption was noted as "complete" at the end of 9 weeks and at 10 weeks the colony count was higher than the initial count (49,500). In 5 of the 7 experiments the colony count at the end of the experiment was higher than at the outset.

Storage under Sand.—Clean sand from Lake Michigan beach was used instead of soil in another series. The sand had a much lower bacterial content than the soil (average 50 per mg.). Storage was carried out at room temperature and the conditions were similar in all other respects with those in which soil was used. The changes in bacterial content ran a strictly parallel course (table 8) with those previously described.

TABLE 8

CHANGES IN COLONY COUNT IN FECES STORED UNDER BEACH SAND AT ROOM TEMPERATURE, TAKING INITIAL NUMBER AS 100

Storage Period	Count	Storage Period	Count
5 days	8,036	8 weeks	709
12 days	8,463	10 weeks	51
26 days	3,145	12 weeks	2
6 weeks	563		

In other experiments the number remained high for long periods. The initial count of the fresh feces in one experiment was 5,400 per mg. and after 17 weeks at the period when absorption had taken place the

count of the sand was 8,000 per mg. In another experiment the number was for fresh feces 8,000 per mg.; after 17 weeks 14,500. Absorption of the feces by the sand occurred just as by the soil. It was usually "complete" to inspection in about 10 to 12 weeks, but was perhaps not quite so rapid as the absorption by soil.

B. coli Count.—In all cases B. coli enumerations were made by use of the Ayers and Rupp medium at the same time and upon the same samples taken for colony counts. The results obtained in the storage experiments precisely duplicated those obtained on fresh feces, no constant relation being found to exist between the total colony count and the numbers of B. coli. The ratio of B. coli to the total colony count on the same sample might vary all the way from nearly 100% to 1% or less.

The results of the B. coli enumerations in themselves are peculiarly interesting. The changes in numbers of B. coli at room temperature storage will first be considered and the effects of the other temperatures taken up later. The following is typical of the 13 experiments of the room temperature series:

TABLE 9

CHANGES IN NUMBERS OF B. COLI IN FECES STORED AT ROOM TEMPERATURE, TAKING INITIAL NUMBER AS 100

Storage Period in Days	Count	Storage Period in Days	Count
3	7,000*	14	530
5	3,730	16	24
7	3,600	18	26
9	2,400	21	24
11	930	23	14

* At the end of the first 24 hours relatively little change had occurred in most experiments; at 48 hours an increase was usually noticeable.

It is evident from this series that the initial multiplication, so characteristic of the total colony count is in most cases chiefly due to B. coli; and that this increase is followed by a decline which brings the B. coli content to a point lower than that obtaining originally. There are, however, two notable deviations from the usual course taken by the total colony count. As shown in the first section of this paper, the total colony count in stored feces may be higher after the lapse of weeks or months than the initial count. The B. coli count, after the initial rise, falls to a relatively low figure and does not rise again under the conditions prevailing in these experiments. In the second place, the fall in B. coli numbers occurs relatively early. In the majority of the 13 experiments, living B. coli were not found in 10 mg. samples of feces after 6 to 8 weeks of

storage. There are irregularities and exceptions, but in general it can be said that after an initial multiplication in feces stored at room temperature, B. coli decreases rapidly in numbers and tends to disappear in a relatively short time. The longest period of survival noted was 12 weeks, 210 per mg., as against 31,000 per mg. in the fresh feces.

Effects of Temperature. Storage at 37 C.—The life of B. coli in feces stored at incubator temperature (37 C.) is quite different from that at room temperature. The initial multiplication takes place earlier, often reaches a very high point within 24 to 48 hours, and then quickly declines, so that in 10 mg. samples the presence of B. coli can no longer be demonstrated.

TABLE 10

CHANGES IN NUMBERS OF B. COLI IN FECES STORED AT 37 C., TAKING INITIAL NUMBER AS 100

Storage Period	Counts	
	Exper. 22	Exper. 29
3 hours	430	1,200
6 hours	1,720	1,200
24 hours	50,180	200,000
48 hours	45,800	223,000
3 days	6,750	61,200
6 days	18	4
8 days	4	..
9 days	9	0 (in 10 mg.)
12 days	1	0 (in 10 mg.)
20 days	(in 10 mg.) 0	0 (in 10 mg. 19 days)

In the six experiments in this series the earliest date at which B. coli ceased to be found in 10 mg. samples is shown in table 11.

TABLE 11

B. COLI NOT FOUND IN FECES STORED AT 37 C. (10 MG. SAMPLES)

Experiment	Storage Period in Days	Experiment	Storage Period in Days
14	7	29	9
16	11	18	8
22	20	31 discontinued after 10 days. B. coli then 47% of initial number	

The actual numbers of B. coli that are reached in the course of the great proliferation that occurs at room temperature and at 37 C. are not very different. The highest number reached (per mg. of feces) at room temperature was 12,250,000 after 10 days' storage, and that at 37 C. was 5,575,000 after 48 hours' storage.

The chief effect on B. coli of storage at 37 C. seems, therefore, to be the hastening of the initial multiplication and of the ultimate destruc-

tion of the organism. The whole process is telescoped and the period of survival of B. coli noticeably shortened.

Storage at 10 C.—The several lots of feces stored at refrigerator temperature (about 10 C.) gave still other results. As compared with storage at room temperature the initial multiplication makes its appearance tardily and the survival period of B. coli is considerably lengthened. The highest count reached (per mg. of feces) was 7,200,000 after 20 days, which is similar to the high counts quoted above for 37 C. and room temperature.

TABLE 12

CHANGES IN NUMBERS PER MG. OF B. COLI IN FECES STORED AT 10 C.

Storage Period in Days	Count	Storage Period in Weeks	Count
Initial	11,000	10	65,000
7	90,000	12	130,000
14	142,500	14	6,800
21	129,000	16	1,090
28	9,000	18	8,000
35	4,500	20	3,500
42	2,250	22	50
54	19,000	23	55

In practically the whole series (8 experiments) B. coli, so far from having disappeared from 10 mg. samples after 2 or 3 weeks, was present in substantially undiminished numbers at the end of 2 or 3 months of storage. The lower temperature retards the multiplication and lengthens the survival period of this organism. In three experiments the numbers of B. coli were considerably higher after 8 weeks' storage than they were initially, and in a fourth they were practically the same as the initial count (96%).

Storage at —11 C.—In the lots of frozen feces stored at —11 C. B. coli were present in 10 mg. amounts up to 11 weeks in one case, up to 13 weeks in another, and in the third experiment it was still present in small numbers (3 per 10 mg.) after 18 weeks.

Storage under Garden Soil.—Multiplication of B. coli in masses of feces stored under soil at room temperature (6 experiments) takes place in much the same way as in uncovered feces, though possibly the numbers reached are not quite so high (maximum in this garden soil series 1,445,000 per mg., as compared with 12,250,000 per mg. in uncovered feces at room temperature, 5,575,000 at 37 C. and 7,200,000 at 10 C.).

The initial multiplication is followed by a decline which seems to be somewhat less rapid than the decline observed in the B. coli content of

the uncovered feces. While after 6 to 8 weeks B. coli was usually absent in 10 mg. amounts in the uncovered feces stored at room temperature, the survival period was approximately twice as long in the lots stored under garden soil (table 13).

TABLE 13

B. COLI NOT FOUND IN FECES STORED UNDER GARDEN SOIL (10 MG. SAMPLES)

Experiment	Storage Period in Weeks	Experiment	Storage Period in Weeks
8	16	25	16
12	12	37	15
20	19	49	10

In several instances B. coli was found in the soil in appreciable numbers after "complete absorption" of the feces had taken place, although the soil did not contain B. coli in 10 mg. samples before the addition of feces had been made. Thus it was noted that the feces were entirely absorbed by the soil at the end of 12 weeks, but the 14 weeks' examination showed B. coli still present (1,789 per mg. or 13% of the original B. coli content).

Storage under Sand.—Initial multiplication, which reached its highest point in exper. 26 (3,050,000 per mg.), followed by a decline, was observed in all the experiments in this series (6) at room temperature. The survival period as indicated by the presence of B. coli in 10 mg. amounts was somewhat shorter than observed in samples stored under garden soil (table 14).

TABLE 14

B. COLI NOT FOUND IN FECES STORED UNDER SAND (10 MG. SAMPLES)

Experiment	Storage Period in Weeks	Experiment	Storage Period in Weeks
19	12	33	8
26	8	39	9
32	10	41	8

B. coli and B. aerogenes Types.—A few observations were made upon the relative frequency of bacteria of the colon type in fresh and stored feces. A number of typical colonies were picked at random from the plates of the Ayers and Rupp medium and, after the purity of the cultures had been determined by replating the individual strains, were subjected to the methyl red test and Voges-Proskauer reaction.[12] The

[12] Rogers, Clark and Lubs: J. Bacteriol., 1918, 3, p. 231.

two main groups (low ratio = M. R. +, V-P —, = B. coli type; high ratio = M. R. —, V-P + = B. aerogenes type) usually present were sorted out according to this method. Colonies picked from plates of fresh feces gave the results shown in table 15.

TABLE 15
PROPORTION OF B. COLI AND B. AEROGENES TYPES IN FRESH FECES

Stool	Total No. of Colonies Picked	B. coli Type	B. aerogenes Type
A	40	31	9
B	10	10	0
C	10	10	0
D	11	11	0
E	10	10	0
Total	81	72	9

The percentage of B. aerogenes in fresh feces is similar to that noted by other observers. As shown in table 15, individual differences exist in the proportion of the two types. Colonies picked from plates after these same lots of feces had been stored at room temperature for from 3 to 16 days gave 44 of the B. coli type and 7 of the B. aerogenes type. It seems probable that no marked changes in the relative abundance of the two types occurs during this brief storage period, but the numbers are too small to warrant definite conclusion. Winslow and Cohen [13] found a relatively greater decrease in the B. coli than in the B. aerogenes type in inoculated water samples stored for long periods. Further observations are needed to determine whether a similar change occurs in stored feces samples.

THE LONGEVITY OF TYPHOID BACILLI IN STORED FECES

The length of life of the typhoid bacillus in feces does not seem to have been adequately studied by modern methods of isolation and identification. Some of the earlier observers who reported long survival periods did not use the criteria for identifying the typhoid bacillus, such as agglutination, that are now regarded as essential.

The following observations have been made under conditions of storage similar to those used in the experiments on the storage of normal feces. A few observations were made upon normal stools to which were added various strains of pure cultures of typhoid bacilli. The longest survival period observed in these experiments was 21 days.

For several reasons the use of artificially cultivated strains cannot be regarded as satisfactory and the observations now to be recorded

[13] J. Infect. Dis., 1918, 23, p. 82.

deal altogether with the storage of the natural stools of typhoid patients and typhoid carriers which were proved by examination to contain typhoid bacilli shortly after discharge.

The Endo medium was used for isolation. It was sometimes supplemented by the brilliant green enrichment method, but in our hands this method has not given any larger proportion of positive results than the Endo medium. Typhoid-like bacilli have been subjected to the usual differential tests: active motility, failure to liquefy gelatin, acid without gas in dextrose broth, slight acidity in indicator milk. Positive agglutination with immune rabbit serum was regarded as an essential character for final identification. All the observations recorded in the table were made on stools stored at room temperature. Negative findings were followed within two or three days by another examination and the search for typhoid bacilli was usually not abandoned until three or four consecutive examinations had failed to yield typhoid bacilli.

TABLE 16

LONGEVITY OF TYPHOID BACILLI IN STORED FECES

Stool from Typhoid Carrier	Longest Survival Period in Days
A	15
B	42
B	52
B	18
C	38
D	5
E	14
F	24
G *	62
H †	10
I	3

* The organism isolated throughout the whole series of examinations from this case was culturally B. typhosus but did not agglutinate with typhoid serum.

† Two cultures obtained on the 11th day of storage were culturally like B. typhosus but failed to agglutinate.

It is evident that there is considerable difference in the vitality of the typhoid bacillus in different stools, but it is not certain how far the difference is due to the constitution of the feces and how far to the biologic character of the specific strains. Variation in bacterial resistance is strongly suggested to one who followed day by day the plates made from the feces. In some instances large numbers are found when the stool is examined soon after discharge, but after a few days repeated search fails to reveal any B. typhosus colonies. In others typhoid bacilli, though present in moderate numbers, may be isolated with relative ease at every examination made during the course of several weeks. The results indicate that a considerably longer survival period

may occur than noted by some previous workers. Park[14] reports a speedy disappearance of the typhoid bacilli from the stools of typhoid patients, usually within a few hours, exceptionally surviving to 10 days. Morgan and Harvey[15] found a somewhat longer survival period (18 days). The experiments of Mair[16] which were made by adding broth cultures of typhoid bacilli to soil are hardly comparable with those here reported.

SUMMARY AND DISCUSSION

The first noticeable bacterial change in normal feces stored under conditions similar to those obtaining in privy vaults, earth closets and the like, is a multiplication of the already enormous number of bacteria present. The number of bacteria in fresh feces cultivable by the usual standard methods has been found to average approximately 75,000 per mg. This number is increased within a few days to many times, sometimes to several hundred times, the original count. It is peculiarly interesting that this initial multiplication is due mainly if not wholly to an increase in the numbers of B. coli. This increase occurs at low and high temperatures (37, 20 ±, 10 C.) and therefore is not to be regarded as due to the temperature change occurring when the feces leave the body. A possible explanation is that an inhibitory influence exists in the lower intestine and that this is manifested even on the characteristic microbe of the intestinal contents of that region. When that inhibition is removed by the discharge of the feces from the body, B. coli multiplies extensively in the congenial soil.

The increase in the numbers of B. coli, which at first is the chief constituent of the "total count," is followed by an irregular and variable decline. Like the multiplication, the decline is more or less rapid according to the conditions of storage temperature. It might be expected, from analogy with other bacterial observations, that at 37 C. not only would the initial multiplication begin earlier, but the decline also would set in earlier and take place more rapidly than at 20 C. This is actually what occurs, the effect being most marked in the special observations on B. coli.

A considerable decline in "total count" from the high point is usually observed, but in many lots of feces it fails to carry the numbers much below or even as low as the initial "total count" of the fresh stool. This

[14] J. Am. M. Assn., 1907, 49, p. 852.

[15] J. Roy. Army Med. Corps, 1909, 12, p. 587; quoted by Chapin: Sources and Modes of Infection, 1916, p. 10.

[16] J. Hyg., 1908, 8, p. 37.

was found true in observations extending over many weeks or indeed several months (e. g., table 5). The persistence of high numbers is not, however, due to the continued longevity of B. coli. This organism undergoes a progressive and fairly rapid decrease in numbers soon after the striking initial multiplication. At ordinary room temperature (20 C.) B. coli usually disappears within 6 to 8 weeks from suspensions containing 10 mg. of feces. At 37 C. its disappearance is still more rapid.

There is, therefore, a multiplication of fecal bacteria other than B. coli in the stored feces. This secondary multiplication appears to take place somewhat later than the multiplication of B. coli itself. The nature of the organisms concerned has not yet been determined, so that the metabiotic character of the phenomenon remains uncertain. The extent to which B. coli is actually displaced by this secondary multiplication is also conjectural.

It has not been thought worth while to determine the date of complete disappearance of B. coli from the stored fecal masses since individual variations render such determinations of little practical significance. The percentage reduction in all cases is so sweeping that demonstration of the occasional survival of a few highly resistant cells would add nothing to our understanding of the conditions. It is known that such survival does occur and its biologic importance is fully recognized.

Covering the feces with garden soil or beach sand did not result in any more rapid disappearance of B. coli than leaving them uncovered, although absorption of the feces by the soil occurred within a few weeks in the usual manner. Indeed, B. coli seemed to persist over a longer period in the few experiments in which garden soil was used than when the feces were left uncovered. A larger series of experiments is needed to determine this important point. In other respects the bacterial changes in feces covered with soil or sand were closely parallel to those observed in uncovered samples. There is the same initial multiplication, followed by a slow, irregular decrease. For months after "complete absorption" has occurred, the original position of the fecal mass is betrayed by an excessively high bacterial count in the soil at that point.

Feces obtained directly from typhoid patients and typhoid carriers and found to contain typhoid bacilli have been stored under similar conditions to those used in the experiments on normal feces. If the average and maximum longevity of the typhoid bacillus (maximum 52 days)

has been found to be somewhat higher than in previous observations, this is probably because the examination of a considerable number of colonies at frequent intervals has increased the chances of finding surviving cells. Individual variation in resistance is probably a factor of importance in determining the survival period. It is evident that the longevity of the typhoid bacillus in the contents of privy vaults is sufficiently great to conform to the epidemiological observations that have seemed to trace typhoid outbreaks to this source. On the other hand, no multiplication of typhoid bacilli in stored feces has been observed and all the evidence points to a destruction of this organism of the same nature as that definitely observed in fecal B. coli, and probably more rapid. That long-unused privy vaults or earth pits are infectious seems in the highest degree improbable.

THE INFLUENCE OF THE SIZE OF THE INOCULUM ON THE INCUBATION PERIOD AND COURSE OF RABBIT SYPHILIS

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Neisser [1] concluded that the number of Spirochaeta pallida in the infecting suspension exerted an appreciable influence, not only on the success of the inoculation, but also on the incubation period of the primary lesion in apes. More recently, Nichols and Walker [2] reported that apparently the inoculation of a suspension rich in spirochetes into the scrotum of rabbits shortened considerably the incubation period of the primary lesion. Brown and Pearce [3] found that the inoculation of a dilute lymph node emulsion of spirochetes into the testicles of rabbits lengthened the incubation period but did not alter the later progress of the orchitis. Chesney and Kemp [4] infected rabbits intratesticularly with graduated doses of the Nichols strain of the organism. They found an inverse ratio between the size of the dose of the spirochete and the incubation period, but observed no marked effect of the number of spirochetes in the inoculum on the subsequent course of the disease. These workers also comment on the desirability of studying the effect of an even smaller inoculum on the incubation period and on the subsequent course of experimental rabbit syphilis. We have conducted a similar study, but have used greater dilutions of the spirochete for our inoculations than did Chesney and Kemp.

METHOD

The Nichols strain of Spirochaeta pallida was used. Group A, consisting of 35 rabbits, was inoculated into the right testis with 0.3 to 0.4 c c. of a suspension containing 2 motile spirochetes per 3 fields. The suspension was made from the testes of a rabbit, the local lesions of which were well marked and progressive. Another lot of 35 rabbits comprising group B was similarly inoculated with 1 c c. suspensions containing so few organisms that dark-field examinations were uniformly negative. Each of these 35 suspensions was made from the lymph node of a rabbit with latent syphilis. There was, therefore, a difference in

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[1] Arb. a. d. kaiserl. Gsndhtsmt., 1911, 37, p. 1.
[2] J. Exper. Med., 1923, 37, p. 525.
[3] Ibid., 1922, 35, p. 39.
[4] Ibid., 1925, 41, p. 479.

the sources of the infecting organisms of these two groups. However, there is no doubt that the significant factor was the difference in the number of spirochetes in the suspensions, since the organisms exist in an active state in the lymphatic tissues of latent syphilitic rabbits.[3]

After the appearance of the primary lesion, i. e., the orchitis, 6 animals of each group were observed weekly throughout the entire course of the local (testicular and scrotal) lesions, and for 3 months subsequent to the healing of the orchitis and chancre. Biweekly Wassermann tests were made on these 12 rabbits during the entire course of the experiment. The Wassermann technic used has been described by Heusinkveld and Carroll.[5]

TABLE 1

VARIATION OF INCUBATION PERIODS OF TWO GROUPS OF RABBITS INOCULATED WITH SUSPENSIONS OF SPIROCHAETA PALLIDA OF DIFFERENT DILUTION

No. of Rabbits		
Group A Receiving Inoculum Containing 2 Sp. p. per 3 Fields	Group B Receiving Inoculum Containing 0 Sp. p. per 50 Fields	Incubation Periods, Days
10	0	10–20
21	0	20–30
4	25	30–40
0	6	40–50
0	3	50–60
0	1	103

RESULTS

1. *Incubation Period.*—The average incubation period of group A, inoculated with the heavier suspension of spirochetes, was 23 days; that of group B, given the more dilute suspension, was 40 days. The individual extremes for the former group were 15 and 35 days, and for the latter, 32 and 103 days, respectively. The difference in the incubation periods for the individual rabbits of the two groups is further shown in table 1.

2. *Subsequent Course of the Disease.*—As has been stated, 6 animals of each group were retained to study the effect of the dilution of the inoculum on the subsequent course of experimental rabbit syphilis. The character and duration of the local lesions; i. e., the orchitis and scrotal ulceration or chancre, were essentially the same in both groups. The lesions progressed to a high degree of involvement, maintained that level for a time, gradually regressed, and finally healed. Chancres developed on the scrotum of the inoculated testis of all of the rabbits of group A which received the heavier suspension of the treponemes, and on the

[5] J. Lab. & Clin. Med., 1924, 9, p. 834.

scrotum of 5 of the 6 rabbits of group B, which were given the more dilute suspension. The Wassermann reaction paralleled the clinical signs closely. It became positive approximately 2 weeks after the onset of the orchitis, ascended to a 3 to 4 plus level, and returned to negative with the disappearance of the lesions in both groups of animals. The average duration periods of the orchitis, chancre, and positive blood Wassermann reaction of the two groups were similar. For group A, the figures were 111, 68, and 83 days, respectively. For group B, the time average intervals were 103, 75, and 79 days. The duration of positive findings for the individual rabbits are given in table 2.

Other foci of syphilitic involvement were likewise similar for both groups of rabbits. Five of the 6 animals of each group, or 83%,

TABLE 2

DURATION PERIODS OF ORCHITIS, CHANCRE, AND POSITIVE BLOOD WASSERMANN REACTIONS OF TWO GROUPS OF RABBITS INOCULATED WITH SUSPENSIONS OF SPIROCHAETA PALLIDA OF DIFFERENT DILUTION

Group	Rabbit No.	Duration in Days		
		Orchitis	Chancre	Wassermann
A	1	156	95	115
	2	77	54	75
	3	119	80	104
	4	67	17	41
	5	112	100	104
	6	135	63	59
	Averages	111	68	83
B	7	112	75	94
	8	75	0	90
	9	119	100	0
	10	100	80	90
	11	98	89	85
	12	116	106	116
	Averages	103	75	79

developed an orchitis of the uninoculated testis. Two of the rabbits of each group developed generalized lesions. One of the rabbits of group A developed an extensive alopecia 270 days after inoculation. The other developed subcutaneous gummas about the hind extremities 112 days subsequent to inoculation. In group B, one rabbit developed multiple gummas about the hind feet and a unilateral keratitis 120 and 164 days after infection, respectively, and the other animal showed numerous gummas about all four extremities, a gumma at the base of the tail, and one on the left upper eyelid 105 days after infection. No clinical evidences of bone lesions were observed, but no roentgenograms were made. The generalized lesions of the two rabbits of group B were in most instances somewhat more severe than those of group A.

DISCUSSION

Chesney and Kemp found that for 0.8 c c. of a suspension containing 5 to 10 spirochetes per field the average incubation period was 10 days; for 0.2 c c., 15.8 days; and for 0.05 c c., 19.8 days. We have found an average incubation period of 23 days for 0.3 c c. of a suspension containing 2 organisms of the same strain per 3 fields. The total number of spirochetes of this suspension was somewhat less than that of the highest dilution used by Chesney and Kemp. The incubation period likewise was greater. With the much more dilute suspension used for the second group in our experiment, the incubation period was much longer; viz., 40 days. Obviously, therefore, there was an inverse ratio between the quantity of spirochetes inoculated and the incubation period of the disease, as determined by the first sign of orchitis.

There was, however, no essential difference between the subsequent course of the disease in the two groups of rabbits. The durations of the orchitis, chancre, and positive blood Wassermann reactions in the two groups were approximately the same, both as to averages and as to the extremes of the individual rabbits. The incidence of orchitis in the uninoculated testis was the same in both groups; viz., 83%. This figure is somewhat different from the results of Chesney and Kemp, who found an incidence of 17% of orchitis in the uninoculated testis in the group of rabbits receiving their highest dilution of spirochetes as compared with 60% and 67% for the other two groups. In our work, the incidence of generalized lesions was similar in both groups; viz., 33%, although as previously stated, the lesions of group B were somewhat more severe than those of group A. However, in view of the variability in individual animal resistance and the restricted number of rabbits used, we doubt that the difference was really significant.

In general, therefore, our results are confirmatory of those already reported by Chesney and Kemp.

SUMMARY

The number of Spirochaetae pallidae in the inoculum influences the incubation period of experimental rabbit syphilis. The smaller the number of infecting organisms, the longer is the incubation period.

The number of spirochetes in the inoculum does not appreciably affect the subsequent course of the disease, inclusive of the Wassermann findings.

WASSERMANN REACTION IN RABBIT SYPHILIS

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Heusinkveld and Carroll,[1] in a preliminary report, described the Wassermann technic employed in the Wisconsin Psychiatric Institute, as applied to experimental syphilis in the rabbit. More recently, we published the results of a year's research on the Wassermann reaction in this disease.[2] We found that the reaction is a valuable diagnostic aid and index of the healing powers of therapeutic agents in experimental rabbit syphilis. There is a close parallelism between the clinical manifestations of the disease and the Wassermann test. The reaction in experimental syphilis of the rabbit is an index of tissue-spirochete interaction, and bears no relation to the question of sterilization of the animal in regard to the spirochete.

The research in this field, lately reported by Takenaka,[3] agrees very substantially with these findings. Chesney and Kemp [4] announce a forthcoming publication which will embody their results with the Wassermann reaction in experimental rabbit syphilis.

In this report we give the results of another year's research with the blood Wassermann in this disease.

PROCEDURE

The rabbits studied were usually inoculated in one testis with Spirochaeta pallida. One group of five rabbits, however, was inoculated subcutaneously at the base of the ear, and another group of six animals was reinoculated in a like manner. The Nichols strain of the organism was used. Weekly examinations of the clinical condition of the rabbits were made. The Wassermann reaction was performed at weekly or biweekly intervals with acetone-insoluble antigen and active serum according to the method of Lorenz as described by Heusinkveld and Carroll. The observation period for most of the rabbits furnishing data for this communication varied from 6 to 12 months. Treatment,

Received for publication, Dec. 24, 1925.

[1] J. Lab. & Clin. Med., 1924, 9, p. 834.
[2] Arch. Dermat. & Syph., 1925, 12, p. 670.
[3] Acta Dermat., 1924, 4, p. 75; Abstr., Am. J. Syph., 1925, 9, p. 403.
[4] J. Exper. Med., 1925, 42, p. 17.

when given, usually consisted of 3 weekly intravenous injections of ¼ or ½ the tolerated dose of the compound for rabbits and was begun during the 8th week of the disease. When lymph node transfers were made, the rabbits receiving the node emulsions were observed for a period of 3 months. If no clinical manifestations of syphilis appeared and the Wassermann remained negative during this time, the rabbit was killed, necropsy performed and a microscopic examination of the testes made.

RESULTS

In our previous report, we stated findings based on a total of approximately 1,800 Wassermann tests performed on an aggregate of 200 syphilitic rabbits. The results of more than 1,400 Wassermann reactions carried out on a group of 150 syphilitic rabbits during the past year are in entire agreement with the conclusions already stated regarding the test in the diagnosis of experimental rabbit syphilis, the gaging of the healing powers of therapeutic agents, and the relation of the reaction to clinical manifestations and sterilization in the disease.

Our belief that the Wassermann reaction in experimental rabbit syphilis is an index of tissue-spirochete interaction was in part based on preliminary results obtained by the castration of several syphilitic rabbits.[2] The 4 plus Wassermann reactions of two rabbits showing only orchitis and scrotal chancre fell to negative within a week following bilateral castration. Castration of an animal with no testicular or scrotal lesions but with a large chancre at the base of one ear, however, did not alter the 4 plus reaction. The drop to negative in the first two cases, therefore, was not due to metabolic or other changes resulting from the double castration, but could only be ascribed to the extirpation of the syphilitic lesions. Recently, a series of 6 rabbits exhibiting pronounced unilateral or bilateral orchitis, chancres averaging 1.5 cm. in diameter, and 2 to 4 plus Wassermanns, were castrated bilaterally during the 5th week of the lesions. The Wassermann reactions of 4 of the rabbits fell to negative one week later. The reactions of the other two animals originally 4 plus, became 2 plus, 1 plus, and then negative during the three successive weeks following castration. The reactions of 3 control rabbits (noncastrated) remained positive until the orchitis and chancre spontaneously healed. Three of the castrated animals were held for 3 to 5 months. No syphilitic lesions appeared and the Wassermann remained negative. The effect of partial excision of the gross luetic lesions was studied in one animal with a primary lesion at the base of the ear,

orchitis and scrotal ulceration, and a 3 plus Wassermann, all of approximately 3 months' duration, as well as gummas about the feet and at the base of the tail of one week's standing. Complete castration was performed, but the Wassermann test remained at a 3 to 4 plus level for the succeeding 2 months. The ear chancre and gummas were then removed. The latter recurred a week later, however, and not until they healed 2½ months subsequently did the Wassermann test become negative.

The dependence of the positive Wassermann reaction of experimental rabbit syphilis on the presence of sufficient spirochete-tissue interaction was further shown by the results of the subcutaneous inoculation of 5 animals at the base of the ear. One and one-half to 3½ months later, the 5 rabbits so infected all developed a papule at the site of inoculation 0.2 to 1.0 cm. in size. In 3 of the animals, the papule remained small and nonulcerative and healed in approximately 5 weeks. No other lesions appeared during this time, and the Wassermann test remained negative. In the other two rabbits, however, the Wassermann test became positive when the primary lesion enlarged and ulcerated and a hematogenous extension of the syphilitic process to the testis and scrotum occurred.

That the lesions or reacting tissues of experimental rabbit syphilis and not the mere presence of the spirochete are responsible for the elaboration of the Wassermann body was also attested by the findings obtained from latent syphilitic rabbits. Lymph node transfers made from a series of 40 such rabbits with no clinical signs of syphilis, were with one exception, positive for the spirochete. The Wassermann reactions of these animals, however, were negative at the time of the transfer.

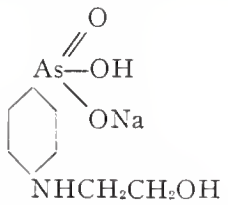
That the presence of sufficient reacting tissue and not the presence of the spirochete is essential to the positive Wassermann reaction of experimental rabbit syphilis was also shown by a study of the Wassermann reaction in rabbits sterilized of the spirochete but with persisting syphilitic lesions. Negative lymph node transfers were obtained from 6 rabbits one week and also 3 months subsequent to the injection of 3 weekly doses of neoarsphenamine, 75 mg. per Kg. of body weight, during the actively syphilitic stage. Despite the absence of the spirochete, the Wassermann reactions of these animals remained positive until the lesions healed one month after the last treatment. Hence, the lesion—i. e., the tissue response to the spirochete—can maintain a positive Wassermann reaction in the absence of the organism.

The relation of the reaction to successful reinoculation was studied. A group of 6 rabbits was sterilized by arsenical treatment; i. e., lymph node transfers made 6 months after treatment were negative for the spirochete in each case. Two weeks after the transfers were made, the rabbits were reinoculated subcutaneously at the base of the ear. Three months subsequent to the reinfection, two of the animals developed a papule at the site of reinoculation 0.7 and 0.9 cm. in size, respectively. The lesion in neither case ulcerated and no lesions appeared elsewhere. Despite the persistence of the papule, the Wassermann reaction remained negative during the 6 months following reinoculation. Three of the animals developed no lesion at the site of reinoculation or elsewhere, but positive lymph node transfers from these rabbits 6 months later proved that the reinoculation was successful. None of the 3 rabbits, however, showed a positive Wassermann test during the 6 months' period. Three months after reinfection, the 6th rabbit developed a large, ulcerated chancre 2.0 cm. in size at the base of the ear, which persisted for 4 months. During this time, the Wassermann test was consistently 4 plus. Thus when the primary lesion of reinfection was nonulcerative and comparatively small or was absent altogether, the Wassermann reaction remained negative. Only in the case where the chancre was large and ulcerative did the Wassermann become positive. Here again, the dependence of the positive Wassermann reaction on the presence of a sufficient amount of tissue-spirochete interaction is evident.

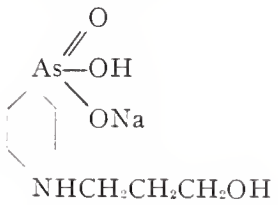
In our earlier publication, we noted a close analogy between the Wassermann reaction of the syphilitic rabbit and that of the luetic human. Recent work has shown another point of similarity. Exceptionally, the Wassermann test is negative in active secondary and tertiary untreated human syphilis. During our studies of the reaction in experimental rabbit syphilis, we have found approximately 350 untreated animals with demonstrable syphilitic lesions of the testes consistently positive to the Wassermann test. However, 3 such rabbits were repeatedly negative to the test. Two of these showed a well-developed unilateral orchitis and scrotal chancre for approximately three months, but the biweekly Wassermann test was always negative during the entire period. The third rabbit developed a bilateral orchitis and chancre lasting 4 months. During the 3rd and 4th months, gummas about the feet and a unilateral keratitis were present. Yet this animal gave a consistently negative Wassermann test. We can not account for these 3 failures to give a positive Wassermann test in spite of the amount of

tissue-spirochete interaction present. For some unaccountable reason, certain animals and certain people do not develop a positive Wassermann reaction even with characteristic lesions present. Of course, it is generally known that there are types of syphilitic lesions which show much less tendency to the production of a positive Wassermann reaction. Thus, the negative Wassermann is common in cardiovascular syphilis.

We have already reported two cases of complete suppression of the appearance of the positive Wassermann reaction where treatment was instituted in the clinically active stage but before the Wassermann reaction became positive. In the later work, 11 more such cases were seen. In two of them, the drug used was neoarsphenamine, in 3 the monosodium salt of 2-p-arsono-anilino-ethanol;[5]



in 3, the monosodium salt of 3-p-arsono-anilino-propanol,[5]



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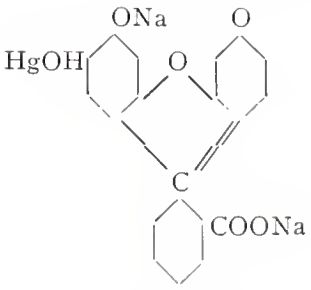

DISCUSSION

These results substantiate our belief that the Wassermann reaction in experimental rabbit syphilis is an index of tissue response to spirochetal influence and bears no relation to the question of sterilization of

[5] Hamilton, C. S.: J. Am. Chem. Soc., 1923, 45, p. 2751.

the animal in regard to the spirochete. In latent syphilis of the rabbit where there is no gross response of the tissues to the spirochete, the Wassermann reaction is negative. As long as active lesions are present, however, the Wassermann reaction is positive, even despite previous sterilization of the rabbit. Partial extirpation of the reacting tissues does not render the Wassermann reaction negative, but a negative reaction results when the gross lesions are completely excised.

However, in order to elaborate serologically demonstrable quantities of the Wassermann body, the lesions must be relatively large and active. When the primary lesion produced at the base of the ear by inoculation or reinoculation was small and nonulcerative, the reaction was negative. If the ear lesion enlarged and ulcerated, or if relatively large and active lesions appeared elsewhere, e. g., on the scrotum or testis, the reaction became positive. The findings of Takenaka [3] are similar to our own in this regard.

SUMMARY

The Wassermann reaction in rabbit syphilis is an index of tissue-spirochete interaction and is not a criterion of the presence or absence of the spirochete.

The Wassermann reaction is consistently positive in about 99% of the cases of active rabbit syphilis following intratesticular inoculation.

The appearance of the positive Wassermann reaction in rabbits affected with syphilis may be completely suppressed by the institution of adequate treatment in the clinically active but prepositive Wassermann stage.

A STUDY OF DÖDERLEIN'S VAGINAL BACILLUS

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Since the pioneer work of Döderlein, little has been added to our knowledge of the vaginal bacillus. The purpose of the study of this bacillus by our present methods, was to obtain a more complete knowledge of the organism.

In 1892, Döderlein,[1] first described the vaginal bacillus as a long, thick, nonmotile, gram-positive bacillus occurring in the normal vaginal secretions. It was aerobic or anaerobic on glucose agar, forming minute translucent colonies like "dew-drops" on the medium. He noted its frequent association (39%) with the yeast fungus. Döderlein considered the antagonistic action of the vaginal bacillus on the growth of staphylococci, and the bactericidal action of the vaginal secretions to be due to the lactic acid produced by the bacillus.

Solomon[2] found that the vaginal bacillus (Döderlein) appears usually about 19 hours after birth and by the end of a week predominates. It is by no means the only inhabitant of the vagina, being 16% of the total flora in 24 hours, and 64% after 10 days.

Since the presence of Döderlein's bacillus is an index of normal vaginal secretions, Apel[3] divided the vaginal flora in pregnant women near term into three grades of purity based on the incidence of the bacillus. He considers the factors that counteract the influence of the vaginal bacillus in favor of pathogenic invaders to be birth trauma, alkaline lochial flow, and a gaping vulva which is a deficient barrier.

The material for our study was obtained from the pregnant women coming to the Cook County Hospital Prenatal Clinic, and from women at term awaiting labor in the hospital. This source of Döderlein's bacillus was thought to be desirable as 50% of women have normal vaginal secretions during the last few weeks of pregnancy (Apel[3]).

Table 1 gives details in regard to the 98 pregnant women whose vaginal secretions were cultured; in 7, cultures were repeated because of interesting findings.

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[1] Das Scheidensekret, 1892.

[2] Ztschr. f. Geburtsh. u. Gynäk., 1923, 85, p. 554.

[3] Arch. f. Gynäk., 1923, 119, p. 115.

This series was especially favorable for the source of Döderlein's bacillus because of the predominance of primiparas. In primiparas the perineum is uninjured, the introitus is well closed, the cervix has not been traumatized by childbirth, therefore the usual conditions favoring infection by pyogenic organisms was absent. Also the majority of the women were at term.

The bacteriologic findings being considered an accurate index of the character of the vaginal secretions, the appearance, amount and consistency were not recorded in this study.

BACTERIAL EXAMINATION

To obtain material for cultures, the vulva was cleansed with 1:1000 bichloride of mercury solution, the labia minora separated and a swab was carefully inserted into the vagina where it was twisted several times before removing. The swabs were inoculated immediately into dextrose broth and then smears were made. Also sterile physiologic salt solution, and solid medium

TABLE 1

STATISTICS OF THE 98 PATIENTS

Race	No	Age in Years	No.	Parity	No.	Month of Pregnancy	No.
White	36	15	1	1	60	Term	74
Black	62	16-20	35	2	15	8	7
		21-25	36	3	8	7	9
		26-30	18	4	3	6	4
		31-35	6	5	4	5	2
		36-40	2	6	1	3	2
				7	2		
				8	4		
				14	1		

were used instead of dextrose broth in series of 25 cases, but the greatest incidence of Döderlein's bacillus occurred when dextrose broth was used, therefore it was used in more than half of the cases studied. The smears were stained by Gram's method. From the liquid medium subcultures were made immediately on blood agar, Endo's medium and dextrose agar plates and repeated in 24 hours.

The first 50 cases were cultivated both aerobically and anaerobically, while the last 48 were cultivated only aerobically. Döderlein's bacillus was isolated in pure culture and its morphology, cultural characteristics, biochemical reactions pathogenicity and immunologic characteristics were studied. Finding that the organism grew poorly on the ordinary laboratory mediums, a medium that produced readily the most profuse growth was sought.

The mediums used were: broth, lactic acid 0.5%, dextrose 1%; agar 1.5%, lactic acid 0.5 and 1%, dextrose 1%; agar 1.5%, lactic acid 1%, lactose 1%; neutral broth, dextrose 1% or lactose 1%; neutral agar, dextrose 1% or lactose 1%; dextrose agar P_H 7.6, and carbohydrate mediums with Andrade's indicator for fermentation reactions. Blood agar plates, Endo's plates and gelatin tubes were made in the usual way. The mediums for the nitrate and indol tests

were made according to the standard methods.[4] For determining putrefactive ability pieces of sterile placenta obtained at cesarean section were put into plain broth.

The results of examinations of the vaginal secretions from the ninety-eight patients are in table 2.

Döderlein's bacillus occurred in 41 instances or 42% on smears, while isolated in only 13 instances or 13% on culture. This difference in incidence can be explained by the predominance and rapid growth of the staphylococcus and bacillus coli. The findings on smears were verified by negative cultural findings of bacillus subtilis which may resemble Döderlein's bacillus morphologically.

Jötten[5] studied and compared B. acidophilus with Döderlein's bacillus and found that there was a resemblance. Therefore a strain of B. acidophilus was used as a control.

TABLE 2

RESULTS OF VAGINAL CULTURES IN 98 PREGNANT WOMEN

Döderlein's bacillus	13	(In smears—41)
Staphylococcus albus	91	
Staphylococcus aureus	37	
Diphtheroids	52	
Yeasts (oidium)	47	
Streptococcus viridans	33	
Bacillus coli	30	
Micrococcus tetragenes	8	
Pneumococcus	2	

As to the morphology of the different strains of Döderlein's bacillus, strain A was 2 to 17 microns in length, strain B, 4 to 14, strain C, 2 to 10, strain D, 2 to 25, and Bacillus acidophilus, 3 to 9 microns. They varied from slender rods to those with tapering ends, except strain B, which manifested a tendency to assume a spiral form. They occurred singly or in chains, were nonmotile, gram-positive except in older forms, when they became gram-negative and were non-spore-forming.

The colonies of Döderlein's bacillus, strain A, on blood agar plates were flat, fimbriated and hairy, 0.05 to 0.2 cm. in diameter. On lactic acid, lactose agar plates they had a definite concentrated center with fibers radiating outward. Similar colonies occurred on all the other solid carbohydrate mediums.

Those of strain B on blood agar plates were small, round, raised colonies, 0.05 to 0.1 cm. in diameter, with a narrow zone of methemo-

[4] Park and Williams: Pathogenic Microorganisms, 1924.

[5] Arch. f. Hyg., 1922, 91, p. 143.

globin. They were small, round, mushroom-like on lactic acid, lactose and lactic acid, dextrose agar plates. On dextrose agar plates the rim of the colonies was rather heavy; the same type occurred on lactose agar and dextrose agar plates, which showed only scant growth.

The colonies of strain C on blood agar plates were small, round, 0.075 to 0.2 cm. in diameter with a narrow zone of methemoglobin. On all the other solid carbohydrate mediums the colonies varied from solid, round, with heavy centers and rims to small centers with hairy radiations. The growth was more luxuriant on lactic acid dextrose and only scant on dextrose agar.

On blood agar plates strain D did not grow. On lactic acid lactose agar and lactose agar plates a luxuriant growth of flat colonies occurred, which were 0.05 to 0.15 cm. in diameter with heavy centers and rims; while on lactic acid dextrose agar and dextrose agar plates the colonies had a heavy center with thread-like radiations.

All the strains produced in gelatin stabs slow, fine filmy growths, but no liquefaction occurred.

The colonies of B. acidophilus on blood agar plates were flat with a hairy margin, 0.05 to 0.2 cm. in diameter. On lactic acid lactose agar plates the growth was luxuriant and the colonies were flat, thin, with irregular, translucent fimbriated margins. On lactic acid dextrose agar, dextrose agar and lactose agar plates they were similar to those on lactic acid lactose agar plates and the growth was just as luxuriant.

On potato a scarcely visible growth was produced by the different strains. Milk was coagulated only by strains A, B and C, while D produced a growth but no change. Putrefactive changes were not produced by any of the strains. Indol was not formed by any of the strains. Nitrates were reduced to nitrites by all of the strains. The reaction was only slight but was probably due to the poor growth in the unmodified medium. The strains grew more luxuriantly under anaerobic than under aerobic conditions. Also the most luxuriant growth occurred in 1% lactose neutral broth, which is therefore the most suitable for culturing the different strains of Döderlein's bacillus.

The fermentation reactions of the different strains of Döderleins bacillus and that of B. acidophilus are shown in table 3.

Based upon the morphological, cultural and biochemical characteristics, stain C came under the classification of Lactobacillus acidophilus, type A, strain A under Lactobacillus acidophilus, type B.[6] Strain D

[6] Bergey, D. H.: Manual of Determinative Bacteriology, 1923.

resembling a diphtheroid came under Corynebacterium segmentosum. Strain B does not resemble any of the organisms[6] described, except that it has the characteristics of the general group of lactobacillus, therefore provisionally it might be called Lactobacillus vaginae.

None of the strains proved pathogenic to rabbits, guinea-pigs or mice, using large doses of 48-hour dextrose broth cultures.

Sixteen strains of the diphtheroids were studied for their reactions in the carbohydrate mediums and their pathogenecity for guinea-pigs, determined by injecting subcutaneously 2 cc. of a 48-hour dextrose broth culture. According to their morphologic and cultural characteristics, they were classified: Corynebacterium xerosis, 4; Corynebacterium

TABLE 3

FERMENTATION REACTIONS

Carbohydrates	Strains of Döderlein's Bacillus				B. acidophilus
	A	B	C	D	
Dextrose	++	++	++	++	++
Lactose	++	++	++	+	++
Saccharose	+	+	+	+	+
Maltose	++	++	++	+	++
Mannite	0	±	0	0	0
Salicin	0	0	0	0	0
Inulin	0	0	0	0	0
Levulose	++	++	++	0	++
Galactose	+	+	+	+	+
Dulcin	0	0	0	0	0
Dextrin	+	0	0	0	0
Raffinose	0	+	0	0	0
Xylose	0	0	0	0	0

hofmanni, 11; and Corynebacterium diphtheriae, 1. Only 2 strains proved to be virulent by animal inoculation. Therefore the incidence of virulent diphtheroids in the vagina is smaller than in the pharynx.[7] The later study was essential to differentiate some strains of the Döderlein's bacillus from the diphtheroids.

Finally, as the results showed a difference in characteristics of the various strains of organisms, identified as Döderlein's bacillus, immunologic studies were then considered for establishing further differentiation or relationship.

Two rabbits were immunized against two respective strains, B and C, by intravenous injections of 8-10 cc. of 48 hour dextrose broth cultures at 3-4 day intervals, each animal receiving 9 injections.

Eight days after the last inoculation the animals were starved for about 15 hours and bled from the heart.

[7] Pilot, I.: J. Infect. Dis., 1921, 29, p. 62.

The development of immunity was tested by means of agglutination, precipitation and complement fixation.

Agglutination Reactions.—Forty-eight hour dextrose broth cultures were used; the cultures being stirred well, allowed to stand until the heavier particles settled and the homogeneous supernatant fluid suspension used in the tests with equal amounts of diluted serum. These were incubated at 37.5 C. dry heat for from 12 to 15 hours, and readings made after 2 and 15 hours.

The results showed that there was greater agglutination with the homologous than with the heterologous strains. One of the strains showed spontaneous agglutination.

TABLE 4

AGGLUTINATION TESTS

Strains of Baeilli	Rabbit Antiserums Against	
	Döderlein B	Döderlein C
Döderlein A	160	1280
Döderlein B	2560	0
Döderlein C	640	Spontaneous agglutination
Döderlein D	1280	80
B. acidophilus	0	0

TABLE 5

PRECIPITIN TESIS

Strains of Baeilli	Rabbit Antiserums Against	
	Döderlein B	Döderlein C
Döderlein A	160	20
Döderlein B	2560	80
Döderlein C	160	160
Döderlein D	0	20
B. acidophilus	0	0

Precipitin Tests.—These were performed similar to the agglutination reactions except that the centrifuged clear supernatant fluid of 48 hour dextrose broth cultures was used instead of suspensions of organisms.

The results here too bear out the fact that there was an immune reaction (precipitation) in higher dilutions with homologous than with heterologous strains.

Complement Fixation Tests.—Antigens for these tests were prepared from 48-hour dextrose broth cultures, removing the broth by 3 repeated washings with sterile physiologic salt solution. After the last washing fairly heavy suspensions were made in sterile physiologic salt solution and allowed to stand. The homogeneous supernatant fluid from each strain was decanted into their respective containers and enough chloroform added to saturate the suspensions. These were set aside for several hours, after which sterility tests were made.

When these proved sterile and after all the chloroform had evaporated the antigens were titrated for antigenic and anticomplementary properties. The tests were made in the usual manner using 5% washed sensitized sheep cells, 2 units of amboceptor, 2 units of complement and ½ of the anticomplementary dose of the antigen. These were incubated at 37.5 C. dry heat, 45 minutes for fixation and 30 minutes for the hemolytic system. Readings were made ½ hour after removing from the incubator and also after remaining in the icebox over night.

The results of the agglutination, precipitation and complement fixation tests showed that although the strains are distinct yet there may be some relationship among them.

SUMMARY AND DISCUSSION

Döderlein's vaginal bacillus was identified in smears in 42%, and cultivated in 13% of the vaginal secretions from pregnant women. Our results do not disagree with those of other investigators except for the low incidence in cultures. The differences in results when marked can

TABLE 6

COMPLEMENT FIXATION TESTS

Antigens	Rabbit Antiserums Against	
	Döderlein B	Döderlein C
Döderlein A	0	+
Döderlein B	++++	±
Döderlein C	0	++++
Döderlein D	0	0
B. acidophilus	0	±

be accounted for by the degree of thoroughness of the study rather than by the method employed, as illustrated by the works of Williams, Fricke, Kanter and Pilot.

Failure to cultivate in a greater number of cases was probably due to more rapid growth of the associated organisms.

The morphology, cultural characteristics, biochemical, pathogenic properties and immunological reactions of 4 strains were studied; three could be classified under the general group of lactobacillus, while one resembled a diphtheroid. Serologically, they are distinct but there may be some relationship.

CONCLUSIONS

The term Döderlein's vaginal bacillus includes a large group of organisms which, though related, have some differentiating characteristics. This fact makes it difficult to classify them.

Among the mediums used the most favorable for cultivating these organisms was found to be 1% lactose in neutral broth.

Lactobacillus vaginae, a provisional name for the Döderlein strain B studied has never been previously described.

The exacting cultural requirements and the incidence of the organisms in the normal vaginas of new-borns and adults are facts upon which the hypothesis is based that the Döderlein's vaginal bacillus has a function in inhibiting the growth of pathogenic organisms by direct action or by maintaining certain conditions in the vagina by its action on the vaginal mucous membrane.

The associated organisms are not so virulent as when they occur in the pharynx.

STUDIES ON ANTHRAX IMMUNITY

1. THE ATTENUATION OF BACILLUS ANTHRACIS BY MEANS OF SODIUM CHLORIDE AND OTHER CHEMICALS

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Ever since Pasteur [1] attenuated cultures of Bacillus anthracis to a sufficient extent so that they could serve as a vaccine against anthrax, the subject of attenuation of that bacillus has claimed the attention of investigators from time to time. Killed cultures of anthrax bacilli have not generally been found to possess antigenic value when used as immunizing agents. Staub and Forgeot,[2] however, reported that they were successful in attempts at immunizing guinea-pigs against anthrax by means of subcutaneous injections of anthrax bacilli killed by alcohol and ether. Urbain [3] in the following year also used ether in killing cultures of anthrax bacilli and states that he successfully immunized rabbits by this means. Aside from these two recent exceptions attenuated cultures and aggressins [4] alone have given results. The economic interest in improved methods of limiting the occurrence of anthrax and the scientific interest in researches in the immunology of the disease as well as in the bacteriology of the causative organism have stimulated active pursuit of further studies in means of attenuating B. anthracis so that cultures may serve as vaccines. The earlier work on attenuation of B. anthracis for use as a vaccine may be found summarized in the literature. Some of the more recent work is presented below.

T. Matsushitka [5] found that cultures of anthrax bacilli maintained for 1½ years on 10% gelatin at room temperature and transferred at intervals of 2 or 3 months had diminished powers for the liquefaction of gelatin. However, the virulence was not decreased. Then Belli [6] found that when anthrax bacilli were subjected to a temperature of —200 C. the virulence of the bacilli was not decreased; and Poppe [7]

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[1] Compt. rend. Acad. d. sc., 1881, 92, p. 666.

[2] Compt. rend. Soc. de biol., 1921, 85, p. 646.

[3] Ibid., 1922, 86, p. 9.

[4] Bail, O.: Centralbl. f. Bakteriol. I. O., 1904, 37, p. 270. Okuda, S.: Ibid., I. O., 90, p. 562. Matsumoto, T.: Ztschr. f. Immunitätsforsch. u. exper. Therap., 1924, 40, p. 402.

[5] Centralbl. f. Bakteriol., I. O., 1900, 28, p. 303.

[6] Ibid., 1902, 31, p. 355.

[7] Ztschr. f. Fleisch u. Milchhyg., 1914, 24, p. 485.

had similar results when he exposed the anthrax bacillus to a temperature of —15° for 2 weeks. Von Székeley [8] found that when the anthrax bacillus was exposed to diffused light at room temperature and under conditions which favored moderately rapid desiccation virulent spores were found after a period of 18½ years.

Scagliosi [9] reported that he had exposed anthrax bacilli and spores on sterilized threads for 10 years, during which time they were subjected to the action of diffused light. These bacilli were found, on inoculation experiments, to be practically as virulent as fresh cultures. Ditthorn [10] reported experimental work in which he inoculated frogs with the anthrax bacillus and found that the bacillus was not attenuated by passage through these cold-blooded animals. Bordoli [11] reported some work in which he had exposed antrax bacilli to a 10% solution of sodium chloride for 1 month. No decrease in virulence was noted from this treatment of the cultures.

While the foregoing experimental work has yielded results of a negative character, a number of other investigators have secured positive results in their attempts to attenuate the anthrax bacillus. The means of attenuation successfully employed by Pasteur [1] was to cultivate the organisms at temperatures of 42 to 43 C. Pasteur found that after cultivation at this temperature for 12 days, the organisms were attenuated to such an extent that they were only occasionally fatal to rabbits. After an additional 12 days of attenuation, the culture was fatal only to young guinea-pigs and mice. Caro [12] found that in freshly drawn milk, anthrax bacilli lost their virulence in 18 hours when held at 37 C., and lost their virulence in 24 hours when held at 15 or 16 C. This result was attributed to the acid formed in the milk, for when magnesium oxide was added and no free acid appeared in the cultures, the bacilli multiplied rapidly and did not lose their virulence.

Galtier [13] found that when anthrax bacilli were placed in pure glycerin they became so attenuated in 55 hours that they were not able to produce infection in guinea-pigs or rabbits. Galtier [14] reported further that when 2 cc. of Lugol's solution were added to 1 cc. of a sporulated anthrax culture, the mixture may be inoculated immediately

[8] Ztschr. f. Hyg. u. Infectionskrankh., 1903, 44, p. 359.
[9] Centralbl. f. Bakteriol., I. O., 1904, 37, p. 649.
[10] Arch. f. Hyg., 1906, 57, p. 313.
[11] Rev. Hig. y Sanidad Vet., 1917, 6, p. 747.
[12] Centralbl. f. Bakteriol., I. O., 1893, 14, p. 398; Chem. Centralbl., 1894. 1, p. 164.
[13] J. M. Vet. et. Zootech., 1902, ser. 5, 6, p. 65.
[14] Ibid., 1903, ser. 5, 7, p. 462.

into rabbits and guinea-pigs without infection resulting, and the treated animals acquire considerable immunity. Subsequently, Galtier [15] produced a polyvalent immune serum against anthrax and blackleg, in which he used cultures of anthrax bacillus treated with Lugol's solution. Again, Murillo [16] tested the effect of diphtheria toxin in the attenuation of the organism in question, and produced immunity in experimental animals treated with the attenuated culture.

In 1914 Henri [17] reported a series of experiments in which the anthrax bacillus was exposed to the influence of ultra-violet rays. She secured considerable modification of morphologic and physiologic characters, which characters were rapidly restored toward normal by passage of the bacillus through guinea-pigs. Complete attenuation was not secured. However, experimental animals inoculated with the treated cultures showed atypical cases of anthrax. More recently Ducloux [18] reported the use of fish-liver bouillon for the attenuation of Bacillus anthracis. In his test on 66 sheep, he found his vaccine to be superior to commercial vaccines for anthrax.

None of the foregoing methods of attenuation of the anthrax bacillus for the preparation of vaccines, has come into wide usage except the method followed by Pasteur and his co-workers. It is to be expected, however, that varying means of attenuation will produce varying effects on an organism. It is conceivable that some of the means of attenuating B. anthracis are more effective in reducing the virulence, and at the same time preserve to a greater degree the antigenic qualities of the organism. For the sake of a greater margin of safety and more uniform success in its use, the desideratum in the case of anthrax vaccines is a means of attenuation which will cause the organism to become avirulent and which will best preserve its antigenic properties for all animals. The Pasteur vaccine does not meet these requirements as fully as might be desired. While persons engaged in the work of disease control have found the vaccination of animals by the Pasteur method a valuable defensive weapon in reducing the losses from anthrax, the Pasteur vaccine has limitations. Some animals do not react to the vaccine and appear to develop no immunity; in contrast, some animals react too severely to the vaccine and develop vaccination anthrax. Furthermore,

[15] Ibid., 1904, 55, p. 705.
[16] Ztschr. f. Hyg. u. Infectionskrankh., 1906, 54, p. 178.
[17] Compt. rend. Acad. d. sc., 1914, 159, p. 340.
[18] Ibid., 1924, 179, p. 510.

the immunity frequently is of relatively short duration; in many cases it appears to persist not over four months.

These considerations have prompted the present studies, which had as their object the search for a vaccine which could be used with a greater degree of safety, which would more uniformly produce protection in vaccinated animals, and which would give an immunity of greater duration than the Pasteur vaccine. The work herein reported is concerned with the successful preparation of a vaccine answering the first of these requirements.

EXPERIMENTAL PROCEDURES

In this work it was attempted to produce attenuated cultures of anthrax bacilli by growing B. anthracis on agar slants to which had been added chemicals in different concentrations. Chemicals chosen for this test were: sodium chloride to represent a relatively nontoxic neutral salt; potassium ferrocyanide to represent a neutral salt with potentially high toxicity to animal cells; copper sulphate to represent a neutral salt with relatively high toxicity to plant cells; sulphuric acid to represent a mineral acid which would not be sufficiently volatile so that appreciable amounts of it would be lost during sterilization or incubation of the medium; and sodium hydroxide to represent a nonvolatile base.

Preparation of Mediums.—The first step in the procedure consisted in determining the maximum concentration at which each chemical could be used in agar tubes without hydrolysis of the agar to a liquid condition, and the maximum concentration which would not completely inhibit growth of the anthrax bacillus. Agar had been chosen as the medium for this series of tests because the surface of the agar slant affords excellent opportunity for observing even slight quantities of growth. Tubes in which the agar became hydrolyzed, either on sterilization or incubation, were omitted from further procedures.

A quantity of standard agar was made and tubed, the tubes containing 5 cc. each. To the tubed agar the chemicals were added in weighed quantities or in measured quantities of controlled dilutions. In no tube was there a mixture of these chemicals, but each tube represented some one of the chemicals noted below, in a definite concentration. Varying amounts of these chemicals were added to different tubes in order that a graded series of concentrations might be secured. The range and degree of concentrations of chemicals was as follows:

$NaCl$: 10, 8, 7, 6, 5, 4, 3, and 2%.
$K_4Fe(CN)_6$: 10, 5, 1, 0.5 and 0.1%.
$CuSO_4$: 10, 5, 1, 0.5 and 0.1%.
H_2SO_4: 2, 1, 0.5, 0.25, 0.10, 0.05, and 0.01%.
$NaOH$: 2, 1.50, 1.0, 0.75, 0.50, 0.25, 0.15, 0.10, and 0.01%.

Standard agar contains 0.5% NaCl; this was taken into consideration in making up NaCl agar tubes.

On adding the chemicals to the agar the tubes were sterilized for 30 minutes at 15 pounds' pressure. Hydrolysis occurred in all tubes where H_2SO_4 or $CuSO_4$ had been added to the agar; and also in those agar tubes containing a greater concentration of NaOH than 0.15. The 0.01% H_2SO_4 tubes, the 0.10% $CuSO_4$ tubes, and the 0.25% NaOH tubes were semisolid on sterilization. They were inoculated with B. anthracis, became liquid on incubation, and no growth was apparent at any time up to 96 hours after inoculation. Thus, they are reported here as omitted from further consideration.

Cultural Procedures.—All tubes which were not completely hydrolyzed were now inoculated with a culture of B. anthracis, and were incubated at 37 C. The culture of B. anthracis which was used for these tests was a virulent spore culture which had been recovered, 10 weeks previously, directly from the heart blood of a guinea-pig dead from anthrax. Observations were made at 24 hours, 48 hours, and 7 days. It was found that complete inhibition of growth occurred in the NaCl tubes containing more than 5%, and in the $K_4Fe(CN)_6$ tubes containing more than 1% of the salt. Some growth occurred in the remaining tubes.

Thus, 5% NaCl, 1% $K_4Fe(CN)_6$ and 0.15% NaOH were found to be approximately the maximum concentrations of these chemicals which can be tolerated by B. anthracis to the extent that growth will occur in their presence. Agar containing these concentrations was used in further cultivation of the organism. Transfers were made every 7 days from the cultures on inhibiting mediums to similar tubes of fresh mediums. Incubation was maintained at 35-37 C.

Table 1 presents observations on the rate of multiplication of the anthrax bacillus on inhibiting mediums as judged by the amount of growth appearing on the surface of the agar slants. These observations were made from the first set of tubes inoculated. After several weeks' cultivation on the selected mediums the cultures grew somewhat better as compared with prepared controls, indicating a degree of accommodation of the bacillus to environment.

Tests of Attenuation.—At the end of 6 weeks of subculturing of the NaCl series of cultures, and the end of the 7th week of subculturing of the $K_4Fe(CN)_6$ and NaOH series, the cultures were tested to determine whether their virulence had in any wise been impaired. A tube of Dunham's peptone solution was inoculated with one loopful of spores of B. anthracis from the last culture on NaCl mediums; another tube of the same medium was inoculated with one loopful of spores from

the last culture on $K_4Fe(CN)_6$ mediums; and a similar preparation was made from the last NaOH culture. A tube of Dunham's peptone solution also was inoculated with a loopful of virulent spores. These were incubated at 37 for 24 hours, when 4 guinea-pigs were inoculated subcutaneously with 0.3 cc. each from one of the above noted peptone broth cultures. All guinea-pigs used in experiments detailed in this paper were half grown and weighed about 250 Gm. The results of these inoculations are presented in table 2. Guinea-pig 1 was inoculated with the peptone broth culture of anthrax bacilli which had been subcultured for 6 weeks on mediums containing sodium chloride; it remained

TABLE 1

AMOUNT OF GROWTH OCCURRING ON MEDIUMS USED FOR ATTENUATING B. ANTHRACIS

Chemical in Medium, %	Growth		
	24 Hours	48 Hours	7 Days
NaCl 10	0	0	0
NaCl 8	0	0	0
NaCl 7	0	0	0
NaCl 6	0	0	0
NaCl 5	±	Delicate	Delicate
NaCl 4	Delicate	Good	Good
NaCl 3	Fair	Good	Good
NaCl 2	Good	Good	Good
$K_4Fe(CN)_6$ 10	0	0	0
$K_4Fe(CN)_6$ 5	0	0	0
$K_4Fe(CN)_6$ 1	±	Delicate	Delicate
$K_4Fe(CN)_6$ 0.5	Very delicate	Delicate	Fair
$K_4Fe(CN)_6$ 0.1	Delicate	Fair	Good
NaOH 0.15	±	Slight	Fair
NaOH 0.10	Slight	Good	Good
NaOH 0.05	Fair	Good	Good

alive and well until a month later, when it died as the result of other procedures. This gave some evidence that the cultures of B. anthracis grown on mediums containing sodium chloride probably had become attenuated. Guinea-pigs 2 and 3 were inoculated with peptone broth cultures of anthrax bacilli which had been subcultured for 7 weeks on mediums containing potassium ferrocyanide and sodium hydroxide respectively, and guinea-pig 4 was inoculated with a peptone broth culture of anthrax bacilli which had not been subjected to any attenuating process. These three guinea-pigs died in less than 48 hours, and on post mortem all presented typical lesions of anthrax; anthrax bacilli were found microscopically and were cultured from the enlarged spleens, which were found in all cases, and from the heart blood of each animal. This indicated that probably no attenuation had occurred in cultures grown on mediums containing potassium ferrocyanide or sodium hydroxide.

Further inoculations were made to determine whether or not attenuation had occurred in the NaCl cultures, and to determine whether the attenuated culture could be injected into guinea-pigs in larger quantities without producing fatalities. A group of 8 guinea-pigs was used for this purpose. The culture used for inoculating the guinea-pigs was in this case also a 24-hour peptone broth culture of sodium chloride attenuated organisms, prepared by inoculating several 6 cc. tubes of the culture medium with one loop of spores each and incubating at 37 C. These cultures were thoroughly mixed and chains of bacilli were broken up by shaking with glass beads. The inoculations were made subcutaneously. Guinea-pigs 5, 6 and 7 received 0.3 cc. each, guinea-pig 8 received 1 cc., and guinea-pig 9 received 3 cc. of the culture.

TABLE 2

TESTS TO DETERMINE THE DEGREE OF ATTENUATION, IF ANY, WHICH HAD FOLLOWED THE GROWTH OF B. ANTHRACIS ON SELECTED MEDIUMS

Guinea-Pig No.	Bacilli Grown in Agar Containing	Peptone Broth Culture, Cc. Injected	Results	
			At 20 Hours	At 48 Hours
1	NaCl	0.3	O. K.	O. K.
2	$K_4Fe(CN)_6$	0.3	Depressed	Dead
3	NaOH	0.3	O. K.	Dead
4	Control	0.3	O. K.	Dead

Two other guinea-pigs, 10 and 11, which served as controls, received 0.3 cc. each of a 24-hour peptone broth culture of nonattenuated B. anthracis, which had been prepared in the same manner. Parallel bacterial counts by the plating method yielded a count of 246,000 bacilli per cc. for the peptone broth cultures of attenuated organisms, and 212,000 for the peptone broth cultures of virulent bacilli. Both cultures showed a density between tubes 1 and 2 of the McFarland nephelometer.

In these tests, presented in table 3, it was found that of the guinea-pigs receiving sodium chloride attenuated cultures, only no. 9, which had received 3 cc., suffered any ill effects. A slight local reaction occurred in guinea-pigs 5, 6, 7 and 8, causing a small edematous enlargement about the size of a pea at the site of the inoculation, which, however, disappeared in 5 days. However, no. 9 was visibly sick a few minutes after the inoculation. On postmortem it showed no lesions of anthrax nor could anthrax be demonstrated microscopically or culturally; the blood coagulated readily. Extensive lesions of pneumonia were present; the animal probably was sick before inoculation, and its death is ascribed to that cause. The control guinea-pigs died in the usual

length of time from the effects of the inoculation, and proved that 0.3 cc. of inoculum should have been a sufficient dose to have produced death had the NaCl cultures not been attenuated.

The NaCl attenuated culture of B. anthracis was now put to an extreme test. One guinea-pig was inoculated with a 9-day agar slant culture of the sodium chloride attenuated organisms. The inoculation was made subcutaneously. While unattenuated cultures of B. anthracis usually kill a guinea-pig in 24 to 48 hours, this guinea-pig lived 23 days, presenting on postmortem examination a typical case of pneumonia but no evidence of anthrax. Twenty-four hours after the injection of the whole agar slant of NaCl attenuated spores, while the animal did not appear to become sick, an edematous area appeared at the site of the

TABLE 3

FURTHER TESTS ON THE VIRULENCE OF NaCl ATTENUATED CULTURES

Guinea-Pig No.	Virulent Culture in Peptone Broth, Cc. Injected	NaCl Attenuated Culture in Peptone Broth, Cc. Injected	Results
5	—	0.3	Nos. 5, 6, 7, and 8 all remained alive and well. Well-defined edema formed in 48 hours at site of injection; disappeared in 5 days
6	—	0.3	
7	—	0.3	
8	—	1.0	
9	—	3.0	Died, 21 hours (pneumonia)
13	—	(1 Agar Slant)	Marked diffuse reaction. Gradually resolved. Remained well
10	0.3	—	Died, 58 hours (anthrax)
11	0.3	—	Died, 40 hours (anthrax)

injection, which in 3 days became 3 inches broad and covered the ventral face of the abdomen and extended in addition to some extent forward on the thorax. This edematous area was approximately ½ inch in thickness and was hard to the touch. It had, however, almost disappeared at the time of death. At postmortem there was slight congestion and a trace of edema still to be noted in the subcutis. The spleen was normal in size and not congested. The blood coagulated well. Smears from the spleen and heart blood were negative. Thus guinea-pig 13 gave further important evidence that this culture of B. anthracis must be considered as being practically avirulent.

Superficially these results might seem at variance with those reported by Bordoli,[11] who did not secure attenuation of B. anthracis when he exposed a culture of the organism to the action of a 10% solution of NaCl for one month. In the work herein reported, however, the experimental procedure was different from that employed by Bordoli. The writer cultivated B. anthracis on agar containing 5% NaCl through

a series of 6 subcultures made at intervals of 7 days. This involved vegetative growth of the organism under abnormal conditions; in the work of Bordoli no vegetative growth occurred.

Immunization of Guinea-Pigs with NaCl Attenuated Cultures.—The next step was to determine whether or not guinea-pigs which had been treated with sodium chloride attenuated cultures of anthrax bacilli would become resistant to injections of virulent cultures. It was not anticipated that a single subcutaneous injection of 0.3 cc. of the attenuated culture would lead to a sufficient degree of immunity so that a guinea-pig would be able to stand subsequent injections of fully virulent organisms. Therefore, guinea-pigs 1, 5, 6, 7 and 8, which had already received one injection of attenuated culture, were given a further subcutaneous injection of 1 cc. each of a 24-hour peptone broth culture of sodium chloride attenuated bacilli (table 4).

Following the initial injection of sodium chloride attenuated cultures, on May 28, all guinea-pigs showed mild reactions which had subsided completely by June 5, or within eight days. But following the inoculations of June 5, marked local reactions occurred. In 24 hours an edematous swelling as large as the end of a thumb had appeared at the site of inoculation in each animal. This doubled in size by the 48th hour after injection. The swellings on nos. 1 and 5 were not quite so extensive as those on nos. 6, 7 and 8. The animals continued to feed, but appeared somewhat depressed. However, no. 6 died 6 days after the second injection; there had been no indication of severe illness. On postmortem of no. 6, there was found an apparently normal spleen, and the blood was well coagulated. However, there was, at the site of inoculation, subcutaneous edema which had a hemorrhagic appearance. Microscopically, smears from the heart blood appeared free from bacilli, and agar cultures from the heart blood remained sterile. The death of this animal was ascribed to an excessively severe reaction to the vaccine.

On June 12 there was to be found a barely palpable cord of swelling at the site of the last injection on guinea-pigs 1 and 5, while on guinea-pigs 7 and 8 there still remained a mass of swollen tissue as large as the end of the thumb. Guinea-pigs 1 and 5 were again treated subcutaneously on this date, with 1 cc. each of a peptone broth culture of NaCl attenuated B. anthracis prepared as before. Guinea-pigs 7 and 8 were not treated, however. This was the last inoculation made on this group of guinea-pigs. They all died before local reactions subsided. The postmortem findings were as follows:

At necropsy no. 1 showed gastric ulcers, the spleen congested and very slightly enlarged. Cultures and smears were made from the spleen and heart blood and from the subcutis at the point of inoculation where some reaction could still be noted. The smears appeared free from bacilli under the microscope and the agar cultures remained negative throughout. No. 5 presented a mass of edema as large as a pigeon's egg at the site of inoculation. Cultures were not made because the interval between death and postmortem precluded the possibility of obtaining pure cultures. Nos. 7 and 8 were found dead June 27, 1925. An abrupt change of weather had occurred and postmortem findings indicated that pneumonia had been the ultimate cause of death in both cases. At necropsy, however, no. 7 also showed marked congestion and slight edema in the subcutis and musculature at the point of inoculation. There was also present a slight amount of cheesy pus at the site of inoculation and the tissue about the abscess was necrotic, a mass about like a large navy bean. The blood was not completely coagulated and was very dark. The spleen was slightly enlarged and showed some congestion, it being red in the center and dark mahogany at the margins. Blood smears from the spleen of no. 7 were negative. Cultures were made from the skin at the site of inoculation and from the spleen. The cultures on incubation proved to be sterile. No. 8 showed relatively less congestion in the subcutis and in the musculature at the site of injection. The entire musculature of the abdominal wall for a circumference of about ¾ inch was necrotic. There was present, where the inoculation had been made, cheesy pus amounting to about ⅓ teaspoonful. The blood was not completely coagulated, and was very dark. The spleen was markedly enlarged and dark mahogany, but blood smears of the spleen showed no bacilli under the microscope. The cultures of the skin and spleen unfortunately were destroyed before they were examined.

This attempt to immunize guinea-pigs against anthrax by use of the NaCl attenuated cultures failed. It has been the experience of other workers that it is extremely difficult to immunize guinea-pigs against anthrax. According to Matsumoto [4] and Okuda,[4] guinea-pigs do not respond readily to immunizing procedures by the formation of antibodies, and they are very apt to die during the process of immunization. In the work herein reported, there probably was not a sufficient interval of time between successive injections; thus, guinea-pigs received inoculations while in the period of sensitization, or the "negative phase." If more time had been permitted to elapse between inoculations the

results might have been more favorable. In support of this assumption, it may be pointed out that mild reactions followed the first injection of vaccine in all cases. Even guinea-pig 8, which received 1 cc. of the peptone broth culture of B. anthracis as a first injection, did not suffer from any marked reaction to that treatment.

Immunization of Rabbits with NaCl Attenuated Cultures.—It was now decided to vaccinate a group of rabbits. In carrying out the vaccination of this group of rabbits, daily weights of the animals were recorded as an indication of the degree of systemic reaction induced.

TABLE 4

TEST OF NACL ATTENUATED CULTURE OF B. ANTHRACIS USED AS A VACCINE ON GUINEA-PIGS. INJECTIONS OF 24-HOUR PEPTONE BROTH CULTURE

Guinea-Pig No.	Cc. Injected				Result
	5/24/25	5/28/25	6/5/25	6/12/25	
1	0.3	—	1.0	1.0	Dead, 6/24/25
5	—	0.3	1.0	1.0	Dead, 6/18/25
6	—	0.3	1.0	—	Dead, 6/11/25
7	—	0.3	1.0	—	Dead, 6/27/25
8	—	1.0	1.0	—	Dead, 6/27/25

Four rabbits from one litter about 3 months old were used in this experiment. Each received a subcutaneous injection on the 26th of June, of ½ cc. of a 24-hour culture in peptone broth of the attenuated culture. Fourteen days later each rabbit received 1 cc. of a similar 24-hour culture, subcutaneously. Eleven days after the second injection, the rabbits were injected subcutaneously with 0.5 cc. of a 24-hour culture of virulent B. anthracis in peptone broth. Two other rabbits received at the same time 0.5 cc. of the same virulent culture, and they served as controls on the severity of the test.

Following the initial injection of the attenuated culture, all rabbits lost some weight. There was, however, no significant loss noted when the second injection of attenuated culture was given. The vaccinated rabbits lost scarcely any weight when subsequently they were injected with the virulent culture. On the whole the injections caused but temporary fluctuations in weight of the animals. At the site of injection of the attenuated culture a swelling, scarcely as large as a pea, followed each administration of attenuated culture, and this swelling disappeared entirely in 3 days' time. When the virulent culture was injected, one of the control rabbits died in 22 hours and the other in 41½ hours, both revealing at postmortem examination typical findings of anthrax, which were corroborated by stained preparations from the spleen in each case. Of the vaccinated rabbits, however, A, C and D showed no reaction beyond a small area of swelling about as large as a navy bean,

while B showed an area of swelling as large as a lima bean. The swellings on A, C and D had entirely disappeared by the 5th day, whereas that on B took a little longer to subside, it being about as large as a pea on the 7th day. Table 5 presents the procedures to which these rabbits were exposed and the results.

The test of the NaCl attenuated culture of B. anthracis proved conclusively that it would serve as a vaccine for immunizing rabbits against anthrax. It is to be noted that rabbit B was a puny rabbit and showed evidences of rickets and never had been in good condition. While this rabbit lost slightly more weight than the others after the first injection

TABLE 5

TEST OF NaCl ATTENUATED CULTURE OF B. ANTHRACIS USED AS A VACCINE ON RABBITS

Rabbit	24-Hour Peptone Broth Culture of NaCl Attenuated B. anthracis, Cc. Injected		24 Hour Peptone Broth Culture of Virulent B. anthracis, Cc. Injected	Results
	6/26/25	7/9/25	7/20/25	
A	0.5	1.0	0.5	Lived; slight local reaction; no illness
B	0.5	1.0	0.5	
C	0.5	1.0	0.5	
D	0.5	1.0	0.5	
E, Control	—	—	0.5	Died, 22 hours. Typical anthrax
F, Control	—	—	0.5	Died, 40½ hours. Typical anthrax

of vaccine this loss also was but temporary; a daily increase of weight occurred after the fourth day following the first injection of vaccine. When the virulent cultures were injected, rabbit B showed a larger area of edema than the others, but this had practically disappeared by the seventh day.

CONCLUSION

This study has yielded results in so far as an attenuated culture has been developed which is of lower virulence than the Pasteur vaccine and hence may be used with a greater degree of safety to the vaccinated animal. Furthermore the attenuated culture, when used as a vaccine, has proven capable of protecting rabbits. Rabbits, however, are known to possess a lower degree of species resistance to anthrax than is possessed by horses, cattle and sheep. Thus a highly attenuated culture could be expected to establish itself and multiply to a sufficient extent when introduced into a rabbit so as to induce a local reaction, and stimulate antibody production. The same culture might be kept completely in check by bactericidal substances when used as a vaccine on

the large domesticated animals; in that case, the highly attenuated culture would be worthless as a vaccine. If organisms contained in a vaccine are unable to sustain some metabolism, the vaccine can induce no local reaction, nor can immunity result.

Should the NaCl attenuated culture which has proven its superiority as a vaccine from the standpoint of safety to the animal be found to be capable of producing a high degree of persistent immunity on large animals, its value would be established. The culture has been used on sheep with success; this will form the basis of the next paper in this series.

SUMMARY

It was found that sulphuric acid and copper sulphate hydrolyze agar when added to this medium, even in such dilute concentrations as would not be expected to inhibit growth of B. anthracis.

The addition of 4.5% sodium chloride and the addition of 1% potassium ferrocyanide to standard agar appears to represent about the maximum concentration of these chemicals which may be used without completely inhibiting the growth of the anthrax bacillus. The growth-inhibiting concentration of sodium hydroxide is about 0.15%.

An increased tolerance to sodium chloride, potassium ferrocyanide and sodium hydroxide could be noticed in successive transfers of the anthrax bacillus, as judged by the production of a more luxuriant growth.

After growing the anthrax bacillus for 7 weeks on agar containing 1% potassium ferrocyanide, and for the same length of time on agar containing 0.15% sodium hydroxide and testing the culture by inoculating guinea-pigs, no decrease in virulence of the organism could be detected.

After growing the anthrax bacillus for 6 weeks on agar containing 5% sodium chloride, marked attenuation of the anthrax bacillus was demonstrated by guinea-pig and rabbit inoculation.

Attempts to immunize guinea-pigs with the NaCl attenuated culture failed. Presumably this was because sufficient intervals of time were not permitted to elapse between injections.

It was found that rabbits could be successfully and safely immunized by the use of the cuture attenuated by growing on 5% sodium chloride agar, so that they withstood the injection of virulent cultures of the anthrax bacillus in quantities which are regularly fatal to normal animals.

SOLUBLE TOXIC PRODUCTS OF THE ENTERITIDIS-PARATYPHOID B GROUP AND THEIR ACTION ESPECIALLY IN RELATION TO BLOOD SUGAR

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Recent bacteriologic investigations of Dick and Dick,[1] Parker [2] and Olitsky and Kliger [3] tend to show that the pathologic action of bacteria such as streptococcus, staphylococcus and B. dysenteriae, which are not usually recognized as toxin producers, can be identified with soluble products which they elaborate. Since suspensions of organisms of the enteritidis paratyphoid B group when injected intravenously give immediate hyperglycemia,[4 5] accompanied later by characteristic morphologic lesions, it was thought that if those phenomena could be elicited with filtrates or chemical compounds obtained from the bacteria it would afford valuable evidence that the pathologic action was in large part due to soluble toxic products.

Gaertner [6] demonstrated that live organisms were not necessary to produce toxic symptoms with B. enteritidis, and Fischer [7] found broth filtrates of the same organism to be toxic. More recently Ecker [8] and Branham,[9] whose papers contain bibliographies of the early literature, have considerably extended our information regarding the properties and action of these toxic filtrates.

The outstanding feature of the early studies is the difference in the toxicity of the products obtained. While much of this variation is undoubtedly due to the presence of peptone in the medium, the varying composition of the culture medium, the degree of aeration and the period of growth are likewise factors in determining the potency of the products.

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[1] J. Am. Med. Assn., 1924, 82, p. 265.
[2] J. Exper. Med., 1924, 40, p. 761.
[3] Ibid., 1920, 31, p. 19.
[4] Menten and Manning: J. M. Res., 1924, 44, p. 675; J. Infect. Dis., 1925, 37, p. 400.
[5] Zeckwer and Goodell: J. Exper. Med., 1925, 42, p. 43.
[6] Korrespondensbl. d. allg. ärtzl. Vereins., Thuringen, 1888.
[7] Ztschr. f. Hyg. u. Infektionskrankh., 1903, 44, p. 243.
[8] J. Infect. Dis., 1918, 22, p. 95.
[9] Ibid., 1925, 37, p. 291.

METHODS

The mediums employed in the present study were extract and infusion broths, with and without the addition of 2% Difco peptone, and an egg broth of the following composition—beef heart 500 gm., lean beef 500 gm., NaCl 5 gm., water to 1000 cc., NaOH to P_H 7.2; whites of 6 eggs and yolks of 2 eggs. The latter was found to be the most satisfactory. One distinct advantage in its favor is the absence of peptone which has been shown by Henderson and Underhill[10] and Kuriyama[11] to produce hyperglycemia on intravenous injection into rabbits. Good aeration was insured by growing the bacteria in about 250 cc. of medium in 2-liter Florence flasks, thus giving a large surface and by occasional shaking. Each flask was inoculated with an 18 hour growth from a hormone agar slant suspended in broth.

The period of growth varied from 24 hours to 3 weeks. The cultures were diluted one-half with distilled water and filtered through Berkefeld N filters. Only those filtrates which were sterile and which gave a negative biuret reaction were injected. In the case of egg medium the large particles were removed by straining through cheese cloth before filtration. The filtrates were injected either without further treatment or there was obtained by the addition of concentrated acetic acid a precipitate which was rapidly dried in a dessicator and powdered. Immediately previous to injection it was redissolved in normal saline or in disodium phosphate, then neutralized by a sufficient amount of acid sodium phosphate, and filtered or centrifuged. Eighty rabbits and 20 guinea pigs were used. Animals were deprived of food for 20 hours previous to an experiment, and blood was obtained from the marginal ear vein. Dextrose in blood filtrates was estimated by the Folin and Wu[12] method. The organisms employed were two strains of B. enteritidis and B. paratyphosus B of animal origin and of human origin.

EFFECTS OF FILTRATES OF B. PARATYPHOSUS B OF ANIMAL ORIGIN ON BLOOD SUGAR CONCENTRATIONS

On intravenous injection filtrates of uninoculated mediums which contained no peptone, produced no appreciable increase in blood sugar during a period of 5 hours. The presence of peptone in uninoculated mediums caused a mild transient hyperglycemia.

With inoculated extract and infusion broths without peptone, filtrates with almost negligible toxicity were obtained. Addition of 2% peptone produced filtrates possessing considerable potency especially in the case of infusion broth, but this latter was generally inferior to the egg medium. Considerable variation in degree of toxicity was found in different samples of the same medium but single intravenous injections of the last three mentioned mediums in amounts of 1 to 3 cc. per kg. of rabbit invariably produced a glycemia.

This glycemia is modified not only by the potency of the product obtained and the amount injected but also by the susceptibility of

[10] Am. J. Physiol., 1911, 28, p. 275.
[11] J. Biol. Chem., 1917, 29, p. 127.
[12] Lab. Manual of Biol. Chem., 1922, p. 253.

individual animals which varies considerably. The results produced by single intravenous injections of filtrates fall into three groups which are typified by the protocols in table 1, which have been chosen as representative.

Group 1.—B. paratyphosus B, of animal origin, was grown on infusion broth plus 2% Difco peptone 4 days. This culture was diluted one-half with distilled water, filtered through Berkefeld N filter and 2.5 cc. were injected intravenously into a rabbit weighing 2750 grams. The variations occurring in the blood sugar are given in table 1. Blood taken at 9 a. m. each morning for hte next 10 days gave a blood sugar between 110 and 130 mg. per 100 cc. of blood. These findings have been duplicated by intravenous injections of suspensions containing 250 to 400 millions of killed B. paratyphosus B of animal origin per kg.

Group 2.—With more potent filtrates the animals succumbed in from ten hours to six days as is illustrated in the two protcools which were selected as typical. The outstanding feature to which attention is called in this group is the antemortem rise in blood sugar. About 50% of the animals receiving filtrates gave reactions of the Group 2 type. B. paratyphosus B was also grown 3 days on egg medium, strained to remove large particles, diluted one-half with distilled water and filtered through Berkefeld N filter. Into a rabbit weighing 2500 grams, 2 cc. were injected intravenously, and the recorded blood sugar values per 100 cc. of blood were obtained.

The terminal hyperglycemia may occur with almost no period of normal glycemia intervening between it and the initial hyperglycemia of which the following is an example. B. paratyphosus B was grown five days on egg medium and filtered through Berkefeld N filter. The filtrate was heated three times at 100 C at intervals of a week. Three cc. were then injected intravenously into a rabbit of 2475 gm. Repeated heatings of samples of this filtrate caused a reduction in the degree of the initial hyperglycemia but this was not a characteristic feature of heating of all filtrates, as will be noted later.

Group 3.—The animals in this group received undiluted filtrates and the cultures from which they were derived were grown for one week or more. With amounts sufficient to kill in 3 to 6 hours the hyperglycemia is of brief duration and is followed by a rapid fall in blood sugar, during which the animal dies. B. paratyphosus B was grown nine days on egg medium and filtered undiluted through Berkefeld N filter. 3.5 cc. of filtrate were injected intravenously into a rabbit of 1750 grams. The sugar estimations are recorded.

Results similar to this were obtained with injections of 2 to 4 billion killed organisms per kg. of animal weight.

Assuming rabbits of similar susceptibility with filtrates of equal potency, the blood sugar reaction depends upon the amount injected. The greater the amount, the more rapid the onset of the rise in blood sugar, the shorter the duration of the hyperglycemia and the more intense the ensuing fall. With a sufficiently potent filtrate this fall in blood sugar may reach the fatal hypoglycemia. With a diluted filtrate a significant hypoglycemia is rarely obtained. In group 2 subsequent to the initial rise the sugar values remain about normal for periods varying

from 1½ hours to 5 days, when an antemortem hyperglycemia is observed.

EFFECTS OF FILTRATES OF B. ENTERITIDIS ON BLOOD SUGAR

The B. enteritidis filtrates were less toxic than the B. paratyphoid B in equal doses. B. enteritidis was grown 4 days on egg broth, diluted one-half and filtered through a Berkefeld N filter; 1.5 cc. of this filtrate were injected intravenously into a rabbit weighing 2250 grams. The blood sugar estimations for five hours are recorded (table 1).

TABLE 1

LOOD SUGAR ESTIMATIONS, MG. PER 100 CC. BLOOD, IN RABBITS AFTER INJECTION OF BACTERIAL PREPARATIONS

Organisms	Material Injected	Before Injection	20–40 Min.	Hours 1	1½	2+	3+	4+	5+	24	48	72	96	120	
B. paratyphosus B (animal) Group 1	Filtrate peptone broth	100	124 131	136	200	238	167	154	152	95	—	—	—	—	
Group 2	Filtrate egg medium	109	131	161	222	333	400	266	250	87	91	125	189	—	Died on 5th day
	Filtrate egg medium heated	83	93 103	113	133 105	108	159	167	182	10 brs. 212	—	—	—	—	Moribund, killed
Group 3	Filtrate egg medium undiluted	105	222	188	—	76 *	49	—	—	—	—	—	—	—	Moribund, killed
B. paratyphosus B (human)	Filtrate peptone broth	100	115	—	145	151	148	126	—	94	115	102	105	182	Died 5 hrs. later
B. enteritidis	Filtrate egg medium	113	133	143	—	160	154	—	121	—	—	—	—	—	
B. paratyphosus B (animal)	Precipitate from filtrate	112	106	200	—	220	286	400	167	—	—	—	—	—	Died following night
B. paratyphosus B (animal)	Reinjected with precipitate	122	142	154	—	117	93	92	—	81	189	—	—	—	Died on 3d day
	Reinjected with precipitate	100	89	—	93	61	57	40 *	—	—	—	—	—	—	Moribund, killed

* Heart puncture.

Blood sugar for the following ten days ranged from 95 to 112 mg. and the animal apparently recovered. With sufficiently large doses the results paralleled those obtained with B. paratyphosus B, and the animals died in three hours.

EFFECTS OF FILTRATES OF B. PARATYPHOSUS B OF HUMAN ORIGIN ON BLOOD SUGAR

Filtrates obtained from B. paratyphosus B of human origin gave, on intravenous injection, reactions similar to those recorded with strains isolated from animals. Two cc. of a Berkefeld N filtrate, from a culture of B. paratyphosus B of animal origin, grown five days in an extract broth containing 2% peptone (negative biuret reaction) were injected intravenously into a rabbit weighing 1750 grams. Blood sugar estimations were made during the following 5 days (table 1).

A few deaths occurring within 3 hours after an injection of filtrates from peptone mediums grown less than 48 hours have been discarded because it was suspected that the toxic effect was complicated with the additive glycogenolytic action of peptone. In these cases subsequent examination of the filtrate revealed a faint positive biuret reaction which had been overlooked in the first test.

AQUEOUS EXTRACTS AND AUTOLYSATES

The effects obtained by injection of filtrates have been duplicated by substances soluble in water or normal saline which were extracted from the bacteria themselves. While autolysis at 38 C for varying periods of time was first tried it was subsequently found that similar results were obtainable by simple extraction of large quantities of organisms grown on agar and filtration through Berkefeld N filter of the extract. Finally, organisms grown in quantity on agar slants in bottles were washed off in 96% alcohol, extracted in alcohol for 48 hours in a Soxhlet apparatus and the residue rapidly dried over sulphuric acid in a partial vacuum. This dried powder apparently keeps indefinitely and saline suspensions of weighed portions of these powders were used in the production of antiserum.

EFFECTS OF PRECIPITATES OF TOXIC SUBSTANCES

The effect of a large quantity of toxic substance is best illustrated by concentrating the toxic substance by precipitation. This is accomplished by adding concentrated acetic acid to the sterile filtrate, centrifuging, and drying the precipitate in dessicator spread in thin films. This is then ground in a mortar and injected as a fine suspension in saline or alkaline phosphate solution. This powder is soluble in alkalies and a 1/15 mol. di-sodium phosphate solution after passage through Berkefeld N filter, when neutralized with 1/15 mol. solution of acid potassium phosphate on injection gives toxic effects analogous to those obtained with the untreated filtrate.

A rabbit weighing 1900 gm. was given intravenously 1.9 cc. of a solution made as follows: A Berkefeld N filtrate of B. paratyphosus B (animal origin) grown six days in egg medium was precipitated with acetic acid, dried and powdered. 0.04 gm. of this powder was suspended in 10 cc. of normal saline. The recorded blood sugar estimations were made. Peripheral blood pressure began to fall about 30 minutes after injection and became progressively lower with each subsequent removal of blood.

EFFECT OF REPEATED DOSES OF TOXIC SUBSTANCE

A rabbit injected with a non-lethal dose requires about three weeks to recover. A less time may suffice to stabilize the mechanism controlling sugar metabolism, since a second injection following the first after a two weeks' interval gives the characteristic change in blood sugar. A second dose injected less than two weeks

after the initial dose giving a cumulative effect ends fatally and is interesting in regard to the hyperglycemia which depends upon the amount of injury persisting from first injection as well as upon the amount of second dose. These different results are contrasted in the next two protocols.

One rabbit used in this experiment had been given broth filtrate (group 1) 13 days previously. A second injection, 2 cc. of a suspension made as follows, was then given. 0.01 gm. of a powder obtained from dried acetic acid precipitate of B. paratyphosus B filtrate was suspended in 5 cc. of 1/15 mol. $Na_2HPO_4.2H_2O$ solution in which it is partly soluble. To reduce alkalinity 5 cc. of a 1/15 mol KH_2PO_4 solution were added just previous to injection. The recorded changes in blood sugar occurred.

This response is typical of Group 2 blood sugar reactions and is in sharp contrast to the succeeding protocol where the second injection follows the first after an interval of seven days.

The second rabbit had been given B. enteritidis filtrate with the reactions previously noted. Seven days later 1 cc. of the suspension of filtrate-precipitate just described was injected. The sugar estimations are recorded.

Blood pressure subsequent to the injection became progressively lower. After the last withdrawal of blood the animal was moribund and was killed. While the initial dose in this case was B. enteritidis toxin followed subsequently by precipitated B. paratyphoid B toxin, similar results were obtained when both injections were of B. paratyphosus B materials.

THERMOSTABILITY OF TOXIC SUBSTANCES

The toxic substance in the form either of filtrate or of precipitate does not readily deteriorate with reasonable precautions and is fairly thermostabile. Boiling for 20 minutes decreases but little the toxic action of the substance. Variability in individual rabbits, even when these belong to the same litter renders difficult any exact comparison of the slight differences in reaction between heated and unheated filtrates.

Two rabbits of the same litter weighing respectively 1350 and 1600 gm. received intravenous injections with a Berkefeld N filtrate of a B. paratyphoid B culture grown for 12 days in infusion broth containing 2% peptone. 1.3 cc. of untreated filtrate, and 1.6 cc. filtrate heated for 1 hour at 90 C. were injected, respectively. The blood sugar rise after the injection of unheated filtrate was somewhat higher than after the heated. The results with 7 pairs of rabbits were in general similar to these described.

In two pairs the hyperglycemia in the animals receiving the heated filtrate was slightly greater than in those injected with the unheated fluid. As already noted repeated heating of certain filtrates tends to reduce their hyperglycemic action but this phenomenon was not obtained with all filtrates.

EFFECTS OF ORAL ADMINISTRATION

In an attempt to find out whether the toxic material was absorbed from the intestines, the filtrate was given by mouth.

With doses of 10 cc., practically no effect was discernable. With doses of 25 cc. of filtrate of high toxicity fed by a dropper 1-2 cc. at a time, the rabbits

died in from 4 to 9 days. No immediate hyperglycemia as found after intravenous injection was observed. That a toxic substance (or substances) in the filtrate acts directly upon the tissue was indicated by the presence of many hemorrhagic erosions (some of which showed ulceration) in the stomach mucosa of the fundus in the region of the greater curvature, generally in a wedge shape. As many as 50 of these areas, varying from 0.5 mm. to 0.7 mm. in diameter have been counted in a stomach and the mucosa has been so eroded that they were visible through the serosa, lying at the extremities of the terminal radicles of the gastric artery. They resemble the hemorrhagic lesions produced in stomach mucosa by pilocarpin. Hemorrhage in the medulla of one or both of the adrenals was not uncommon. As the lesions elsewhere than in the stomach are observed some days after the injection it is not clear whether they are due to the direct chemical action of the toxin or are foci of infection set up by lowering the animal's resistance.

SYSTEMATIC MANIFESTATIONS

Symptoms were fairly characteristic and constant. Immediately following an injection the animal became quiet and there was an increased rate of respiration. If the dose was large the blood pressure became low as was evidenced by the difficulty in obtaining blood from the ear veins, there was prostration and urine was voided. After moderately large doses the rabbits refused food and unlike animals suffering from a spontaneous infection, diarrhea was absent. With substances derived from B. enteritidis and especially with suspensions of precipitates, paralysis of the hind legs occurred. This appeared as early as 15 minutes after an injection, but more commonly in from 2 to 3 hours. Paralysis was not common with paratyphoid toxic substances. In none of the experimental animals has a glycosuria been observed.

PRODUCTION OF ANTISERUMS

Although suspensions of precipitates, and residues after alcoholic extraction of B. paratyphosus B as well as enteritidis were employed for the production of antiserums the B. paratyphosus B products were so toxic that the injected rabbits invariably succumbed. As this result was similar to previous experience with the use of suspensions of killed paratyphoid bacteria, experiments with the products of this organism were discontinued. Enteritidis antiserum of high titer was readily obtained in the usual manner by the use of the saline suspensions of precipitates obtained by adding acetic acid to the enteritidis filtrate.

MORPHOLOGIC LESIONS

At necropsy there were found both in rabbits and guinea pigs many of the lesions obtaining with B. paratyphosus B infections in the human. The most characteristic of these were focal necrosis of the liver and

small ulcerations in the lymphoid tissue of the intestines. These lesions, demonstrable both grossly and microscopically, owe their origin to the endothelial cell on which these poisons exert a selective action causing them to swell and become more permeable. These cellular changes are responsible for the vasodilation observed in animals dying during the more acute reactions. As with injections of live or killed organisms the entrance of the toxic substances into the circulation was followed rapidly by an alteration and subsequent lysis of many red blood cells. This was accompanied by a marked phagocytosis of these damaged cells and of freed hemoglobin pigment by endothelial cells lining the sinusoids, and occurring free in the lymph spaces throughout the lymphoid tissue and especially in the spleen. The Kupfer cells of the liver also became loaded with ingested hemoglobin. When a large amount of toxin was injected excessive deposits of brown pigment occurred in the hepatic cells. In animals dying three to five days subsequent to the injection considerable swelling of the endothelial cell could be seen in various tissues and was distinct in the capillaries of the lung. In the lymphoid areas of the appendix one observed not only swelling and mitotic activity of the endothelial cells in situ, but a marked assembling within the sinusoids of free endothelial cells which were so distended with phagocyted erythrocytes as to be of a diffuse golden color. Frequently these congregated in such excessive numbers as to block the lymph spaces and they then underwent disintegration. It has not been possible to determine whether this process was hastened by thrombotic changes or not. Following the dissolution of the endothelial cells, polymorphonuclear leukocytes collected and a small abscess was formed which appeared as a white area varying from 3 to 7 mm. in diameter on the outer wall of the large gut. The formation of these ulcerated areas in the appendix and large intestine was more marked in animals which had been given toxic products than in those infected with the paratyphoid B organisms. In the rabbit, at least, this intestinal lymphoid tissue offers a prolific source for the production of endothelial cells which are actively produced by mitotic division in the wall of the sinusoids. The pathogenesis of the areas of focal necrosis in the liver was similar to the formation of abscesses in the lymphoid tissue. At points irregularly distributed along the wall of the liver sinusoids, gradually increasing numbers of endothelial cells collected. These cells not only infiltrated the hepatic tissue but formed a mass of varying size in the sinusoid. Frequently interlacing threads of fibrin could be distinguished. As the endothelial cells increased in

numbers both they and the hepatic cells underwent degeneration and were replaced by polymorphonuclear leukocytes. The rapidity with which this replacement occurred apparently depended upon the acuteness of the process and the degree of phagocytosis which had been exercised by the endothelial cell. The initial phase of this process in the experimental animal is undoubtedly the injury to the wall of the liver cord, rather than the plugging of the sinusoid by collections of endothelial cells. In some areas lack of evident morphological change in the endothelium suggested direct injury to the hepatic cell itself because of lack of complete endothelial covering of the liver cord. Another change in the liver was vacuolization of the hepatic cells. This reached a maximum in those animals where cumulative effects of the toxins had occurred owing to a dose having been repeated after a short interval. Practically no cytoplasm remained within the cell which appeared to be composed of huge vacuoles. All animals showed biliary cirrhosis of varying intensity and in different stages of development.

The spleen gave evidence of destruction of Malpighian corpuscles and pulp in varying degree.

In the kidney, granular degeneration and occasionally complete necrosis in the proximal convoluted tubule and ascending limb of the loop of Henle was evident as well as degenerative changes of the glomerulus. Coagulation necrosis of the medulla and zona glomerulosa of adrenal similar to those already described in spontaneous paratyphoid infection were likewise observed. Hemorrhage in the medulla of adrenal occasionally occurred.

In the pancreas of rabbits dying with acute symptoms no definite hydropic degeneration was observed in the islets but the beta cells appeared shrunken and often showed pyknosis of nuclei. In a few rabbits of group 2 where death occurred several days after the injection of toxic material, hydropic degeneration was present. In guinea pigs receiving lethal doses there was very readily produced in 12 to 48 hours marked shrinking of the cytoplasm and pyknosis of nucleus of beta cells. With less toxic filtrates hydropic degeneration developed in 4 to 7 days. In animals of group 3 and those of group 2 dying within 15 hours after an injection vasodilation and edema was present in the lung. Capillary thrombosis was not observed, although in two animals many of the capillaries were plugged with masses of fused erythrocytes, which were negative for fibrin with Weigert's special stain. In the more chronic reactions little engorgement of the vessels was noted.

The sequence in the development of the hemorrhagic erosions of the mucosa of the stomach was an initial swelling of the parietal cells accompaned by an enlargement of the peptic cells. The swollen peptic cells in the gastric crypts and necks of glands first underwent dissolution, leaving free parietal cell in the latter situation. This disintegration extended progressively through the body towards the fundus of the gland at which time the parietal cells likewise began to show degenerative changes. Finally, there was a complete loss of all glandular tissue in scattered areas with only thin lines of stroma outlining the position of the gland and a rupture of the terminal radicle to the involved area. This rupture seemed to be accelerated by previous pathologic change in the endothelial lining of the capillaries.

Another interesting lesion was the perivascular round celled infiltration and the marked degeneration of the ganglion cells in the cerebral cortex and cerebellum. This was more marked following injections of B. enteritidis than of B. paratyphoid B toxins.

In evaluating the action of these toxic substances in the pathogenesis of the morphological lesions described, accelerative effects due to possible exacerbations of latent spontaneous infections arising from lowered resistance occasioned by the injection must be borne in mind. The diarrhea and bronchial infections so characteristic of the spontaneous infection were not present.

DISCUSSION

Glycemic responses to intravenous injections of organisms of the enteritidis-paratyphoid B group so closely resemble those obtained from injections of soluble toxic substances derived from these bacteria that one is justified in believing that the active principle in both cases is identical. Allowing for varying susceptibility of the rabbits used the results obtained are directly proportional either to the number of organisms or to the amount of the toxic substance injected. The changes in blood sugar occurring with varying dosage are complex, as indicated by the variety of curves obtained and undoubtedly many factors are concerned in their production. Subsequent to single injections a series of reactions are observed which show all gradations between a mild transient hyperglycemia followed by speedy return to normal and complete recovery and a rapidly developing intense hyperglycemia succeded by a marked hypoglycemia which ends in death in three to six hours. The reactions occurring between these extremes have an added feature, namely a terminal hyperglycemia. Hypoglycemia

may or may not then be present. The hyperglycemia immediately following the entrance of the toxic substance into the blood stream undoubtedly arises from the sudden release of glycogen from the liver and possibly muscle. This assumption is borne out by the pathological changes found in the hepatic cells and by the lack of demonstrable glycogen in the liver as was noted by Zeckwer and Goodell.[5] Likewise McCullough and O'Neill [13] estimate that the similar increase in blood sugar in anaphylactic shock approximates that produced by an injection of glucose equivalent to the glycogen content of the liver. The hepatic cells are rendered more vulnerable to the entrance of these poisons by swelling and increased permeability of the endothelial covering of the liver cords. The explanation of the mechanism underlying the hypoglycemia and the terminal hyperglycemia is not clear with the present available data. Separation from compounds altering blood sugar of constituent elements, producing only a hyper or hypoglycemia as obtained by Dublin and Corbitt [14] with vegetable extracts has not been attempted. It is possible that the hypoglycemia occurring in rabbits receiving a single injection owes its origin to a release of insulin due to pathologic change in the islets of Langerhans, as one frequently sees in these structures pyknosis and shrinking of the beta cells, but this explanation does not seem to suffice for the hypoglycemia without a preceding hyperglycemia such as occurs when a second injection of toxic material follows the initial one at a short interval. Little histologic changes can be detected in the islet cells of such animals.

The terminal rise in blood sugar probably represents a final release of residual glycogen of the muscles, or a disruption of the synthetic mechanism of glycogen arising from depletion of insulin. The histologic appearance of the liver precludes a second hepatic glycogenolysis in those animals where the terminal hyperglycemia is separated from the initial hyperglycemia by an interval of one and a half hours. Moreover, the terminal hyperglycemia lasts a long period, 4 to 24 hours in contrast to the transient primary hyperglycemia of ½ to 2 hours. Pathologic change is not limited to liver but widespread. Morphologic lesions of varying intensity occur in all organs but the most marked alterations are observed in erythrocytes, liver, kidney, lymphoid tissue and adrenals. Endothelial lining of lymph spaces shows a more intense reaction than in that of the blood vessels. The widespread distribution together with the degree of the pathologic change and especially the early alteration in

[13] J. Infect. Dis., 1925, 37, p. 225.
[14] J. Metabol. Res., 1923, 4, p. 89.

endothelium indicate that a direct chemical action on the protoplasm is mainly responsible for the glycogenolysis although stimulation of the sympathetic nervous system as postulated by Zeckwer and Goodell [5] may also be a factor.

The absence of glycosuria, coincident with a significant hyperglycemia, depends upon the raising of threshold for glucose, this latter being an expression of the injury to the secreting unit of the kidney. Lesions are especially evident in tubules and occur early. The condition is probably similar to that noted in peptone hyperglycemia which Kuriyama [11] has attributed to lack of permeability of kidney.

Hyperglycemia secondary to glycogenolysis is not peculiar to the bacteria and soluble toxins of the enteritidis paratyphoid B group but has been obtained by Zeckwer and Goodell [5] with many intestinal bacteria, with peptone by Kuriyama [11] and by Henderson and Underhill,[10] with histamine by Chambers and Thompson [15] and in anaphylaxis by Zeckwer and Goodell [5] and by O'Neill and McCullough.[13] The wide variety of these glycogenolytic agents and the complexity of many of them render difficult the isolation of any chemical group as the causative factor. The toxic substance (or substances) derived from the enteritidis-paratyphoid B group is soluble in water and thermostabile. It can be precipitated by alcohol and by concentrated acetic acid. Continuous heating for 1 hour at 90 C produced almost no appreciable decrease in toxicity. It has not been definitely determined whether the toxic substance is a true soluble toxin or is set free only through disintegration of the cell body.

SUMMARY

Soluble toxic substances derived from organisms of the enteritidis-paratyphoid B group produce alterations in the blood sugar and morphologic lesions which are identical with those produced by injection of the living or killed bacteria.

On intravenous injection of the toxic substances a series of blood sugar curves are obtained which are proportional to the amount of the substance injected. The characteristics of these curves, which have been divided into three groups, are discussed.

A second injection after an interval of less than two weeks causes modifications of the hyperglycemic and hypoglycemic curves obtained with a single injection.

The toxic substance is thermostabile, soluble in water and can be precipitated by alcohol and concentrated acetic acid.

[15] J. Infect. Dis., 1925, 37, p. 229.

THE ORGANISM OF ACTINOMYCES-LIKE TONSILLAR GRANULES

ONE PLATE

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The actinomyces-like granules found in tonsils have been studied and the literature reviewed by David J. Davis.[1] He describes them as small, definite, grayish or grayish yellow bodies or granules, with a foul odor, rather brittle, and showing microscopically a raylike structure resembling in some respects the granules of actinomyces. Each granule is made up of a central shaft of mycelial filaments about which are arranged large numbers of elongated bodies or bacilli. Davis compares these forms to "test-tube brushes." No true branching was observed. Associated with the bacilli and filaments were cocci and spirilla. The bacilli were weakly gram-positive. The bacilli isolated by Davis were never motile, and in cultures did not form colonies or granules that resembled those in the tonsils. Intraperitoneal injections of granules in half grown rabbits produced in 8 days, abscesses in which the bacilli were arranged in clusters that were never so dense, nor so definitely raylike as the original granules.

Davis and Pilot [2] have observed similar granules having a radiate filamentos structure, about teeth with tartar deposits. In the hyalinized epithelium of pharyngomycosis Davis and Hall [3] have described mycotic masses of filaments, fusiform bacilli, spirilla and cocci, the structure of which seems identical with the tonsillar and dental actinomyces-like granules.

The organism studied here was isolated from tonsillar granules that were yellowish, about 1 mm. in diameter, and similar in every way to those described by Davis.[1] Smear preparations showed many thick bacilli with rounded ends, filaments, spirilla mostly tightly waved, and cocci. Some very short spirilla appeared to have bacilli attached to one end. In the dark field the bacilli were actively motile with a progressive, circular, wavy, and trembling motion. The spirilla showed generally a progressive motion and a violent lateral vibration extending from one end of the body to the other. Sections of the granules stained with

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[1] J. Infect. Dis., 1919, 14, p. 144.
[2] J. Am. M. A., 1922, 79, p. 944.
[3] J. Infect. Dis., 1924, 34, p. 203.

Giemsa's stain showed bundles of filaments with the bacillus-like forms radiating from them, and starlike forms with dots inside which might be the filaments in section (fig 1).

The bacillus-like organism is difficult and slow to grow. Two weeks were required to get the original culture in which rods, filaments, spirilla and cocci were found. The rods grew only anaerobically at 36 C. in ascites fluid tissue medium, sheep blood agar, and ascites phosphate agar, and broth, and not on ordinary culture mediums. Herrold's dibasic sodium phosphate 1% agar was made with Bacto beef and peptone and 0.1% dibasic sodium phosphate, adjusted to P_H 7.4-7.6, then to two parts of this agar was added one part of ascites fluid. The cultures were made anaerobic by the use of pyrogallic acid and sodium hydroxide.

After the first generation growth was observed often after 4 to 8 days' incubation, occasionally after 24 hours. Sometimes the cultures would appear dead for several weeks and then begin to grow.

A pure culture of the rods was obtained from a thick cream colored, moist colony about 2 mm. in diameter on ascites phosphate agar. On blood agar the colonies were pinpoint and colorless. The organism grew best in the fluid of condensation and only occasionally produced colonies on the surface. The growth in fluid medium was flocculent but no balls were definitely demonstrated. A little gas was generally produced but no odor. Only a few experiments were made with this organism on account of the difficulty in cultivating it.

The body of the organism is weakly gram-positive, the granules inside definitely so. The organism is not acid-fast, and stains with ordinary stains. By Giemsa's method the cytoplasm stains light blue while the chromatin in granules, bands, and spirals, stains a deep red.

The organism is pleomorphic. The earliest and most frequently observed form is a short rod with square or round ends from 1 to 4 microns in length and about 1 micron in width. In films these rods contain one or more deeply stained chromatin granules. The smallest form is a single granule surrounded by cytoplasm. These bodies may be gonidia. In older cultures are long filaments straight or wavy. No branching is observed but budding of the deeply staining granules occurs. After a new rod is formed, it lies parallel to the rod from which it grew. Sometimes three or four short forms are seen side by side (fig. 5). Some rods appear to divide by snapping, the distal ends then approximating each other giving a parallel arrangement. This method of division has been described by Hall [4] for diphtheria bacilli. Among the rods are sometimes seen many faintly staining bodies. Some organisms divide

[4] J. M. Research, 1902, 7, p. 115.

transversely, sometimes at the deeply staining body which appears to divide and separate. Chains of rods are also found.

The arrangement of the rods in rosette and test-tube brush like forms was seen in both blood agar and ascites phosphate agar cultures. The simplest star has four rays. In some clusters of 4, a round body is seen at the central end of each rod, in others the rods radiate from one or two round bodies (fig. 2 and 3), or from a circular space from which a round body might have disappeared. The latter form is common in older cultures while in the earliest cultures nearly all stars have a central body. Some of these bodies are surrounded by a relatively large amount of cytoplasm, others consist of one large and many small bodies surrounded by cytoplasm or of an oblong mass of chromatin 1 to 4 microns in diameter. The gonidia-like bodies within the rods are often equidistant from the center giving a symmetry suggesting growth and development rather than agglomeration. A fully formed rosette consists of tightly packed rods radiating from a common center. The brush-like forms appear to grow from filaments which contain gonidia-like bodies. Inside of some brush forms are one or more filaments, in others one filament can be seen with an empty space by its side, or both filaments are gone leaving a clear space or faintly staining dots (fig. 4). The rod forms radiating from the filament lie parallel to one another, and often their granules are in the same relative position in each as if they had all grown out at the same time. Some of the brushes have a star form at one end.

In ascites fluid kidney tissue medium, made anaerobic with oil, no stars were seen, but many long filaments, both wavy and straight, in which the chromatin is often arranged in a spiral form. This spiral arrangement is most frequently seen in short rods lying parallel and attached to the filaments. In some, the spiral bodies appear partly inside and partly outside the filament.

There was no indication that the spiral arrangement of the chromatin entered into the formation of a zygospore as described by Mellon.[5]

After nine months' isolation typical spirilla-like forms were occasionally observed in the fluid of condensation of blood agar cultures after 7 days incubation. Their relation to the bacillary forms has not been determined, but merits further investigation.

Flagella may be demonstrated on the ends and sides of the rods and filaments by flagellar stains.

In the dark field young cultures show the rods, chains of rods, and filaments flexible and actively motile. The motion is progressive, circular, whirling, and tumbling. Stars of four rays have great activity but

[5] J. Bact., 1925, 10, p. 481.

fully formed rosettes and brushes are not motile. Flagella are not seen in the dark field, but preparations from ascites phosphate agar cultures 7 or 8 days old show long, thick, regularly waved, spirochete-like spiral forms that are rigid and not motile (fig. 6). They are single or in tangled masses, or may be seen coming from the rosettes or attached to short rods. They are stained only by flagellar stains. These spiral forms appear to be similar to those described by Koga and Otsuba [6] in anaerobic cultures of motile organisms in mediums containing fresh proteins. These authors consider them an unusual development of the flagella or parts of the bacterial bodies. Similar flagellar forms have been seen in the dark field by Neumann.[7]

Sterile hanging drop preparations, sealed with paraffin, were studied for periods of 6 hours on the warm stage. Budding was observed from the end of a short, flexible, motile filament. The end first appeared swollen and fuzzy and finally a short rod was seen to form on the side of the filament and to move a little beyond its end. The central round body in the rosettes is distinct in hanging drop preparations and many small dots are seen inside the brush forms. Before dividing, the organisms appear to become more motile. An irregular granular body was seen to send out buds irregularly. These became long and slender and an irregular star was formed. Small black dots were seen to appear in the rods.

Short motile rods were seen to attack filaments, move off and again to attack, sometimes remaining firmly attached. This is probably conjunction as described by Löhnis.[8]

One cc. of a 4 day growth on blood agar, containing star forms, was injected intracutaneously into a rabbit, but without any apparent effect.

DISCUSSION

Mary Hefferan [9] has described the formation of rosettes or asters in a culture of an aerobic motile bacillus (B. rosaceus metalloides). The bacilli radiated from a common center but no central body was observed. She studied the organism by the hanging block method and was inclined to think that the rosette was formed by the bacillus dividing transversely, then slowly turning until it formed an angle with the other bacillus, and finally separating at the end. She considered the rosettes too perfect for merely passive agglomeration.

[6] J. Infect. Dis., 1919, 24, p. 56.
[7] Centralbl. f. Bakteriol. I. O., 1925, 96, p. 250.
[8] Studies upon the Life Cycle of the Bacteria. Part 1. 1921, pp. 197.
[9] Centralbl. f. Bakteriol., II, O., 1902, 8, p. 689.

Jones [10] described an aerobic motile water organism which formed rosettes which she believed were produced by the uniform grouping of the descendents of a single cell, and were not due to agglutination. The rosette appearance seemed to be favored by anaerobic conditions. The single polar flagellum of each organism pointed toward an unseen center. The flagellum readily took ordinary stains.

Fox [11] observed the formation of a feather or a test-tube brush-like arrangement of an aerobic non-motile bacillus (Mycobacterium plumosum). The bacilli were arranged radially about a center in which no structure was observed. Double rows of parallel rods seemed to branch from an axis in which no structure could be surely seen, although between the rows there was a suggestion of a thread.

Löhnis and Smith [12] explain the formation of star-like forms by the simultaneous growing in different directions of several gonidia in the same cell or in its liberated granular content. This is seen in B. radiobacter. Star-like upgrowths of slender rods from the typical large globular azotobacter forms are also described by these authors. They believe that the star forms may also develop from symplasm.

In my culture the rosette arrangement around one or more central bodies resembles the radial arrangement of "merozoiten" about a resting body in Aggregataria described by Lüke [13] and others.

From the study of stained specimens and hanging drop preparations I am inclined to think the rods form by the budding of gonidia either inside the organism or after their liberation, so that when the gonidia in a filament grow out, a test-tube brush form is produced; when a free gonidium develops a rosette is formed. Further study is necessary to settle this question.

The production by this organism of the test-tube brush-like and rosette forms seen in actinomyces-like granules indicates their significance.

SUMMARY

From an actinomyces-like tonsillar granule a weakly gram-positive flexible motile anaerobic organism has been isolated that produces in pure culture rosettes and test-tube brush-like forms similar to those seen in the original material.

[10] Centralbl. f. Bakteriol., II, O., 1905, 14, p. 459.
[11] Centralbl. f. Bakteriol., I, O., 1913, 70, p. 143.
[12] J. Agric. Research, 1916, 6, p. 675.
[13] Arch. f. Protistenk, 1904, 4, p. 88.

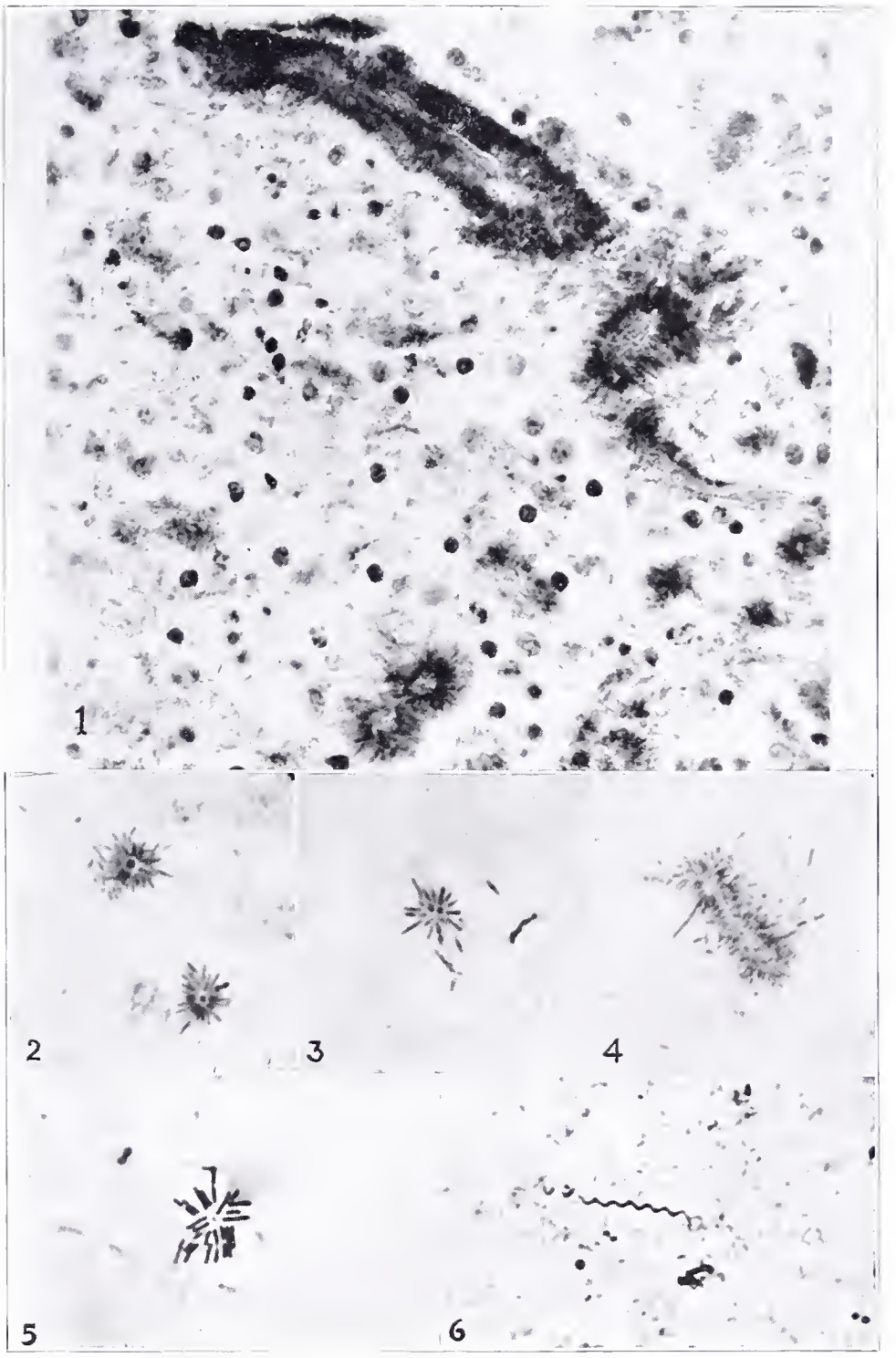

Fig. 1.—Section through tonsil granule. Giemsa's stain; 1: 700.

Figs. 2, 3, 4.—Four days' growth in fluid of condensation of sheep blood agar. Giemsa's stain; 1: 1,200.

Fig. 5.—Six days' growth on sheep blood agar. Carbol-gentian violet; 1: 1,200.

Fig. 6.—Seven days' growth in fluid of condensation of ascites phosphate agar. Flagellar stain; 1: 1,200.

THE INTRACELLULAR PROTEINS OF BACTERIA

1. GLOBULINS

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The problems of structure, physiology and biochemistry in the various fields of biology do not become proportionately simpler as the organisms approach microscopic size. Elaborate researches to ascertain the fundamental principles of nutrition, infection and resistance have failed sometimes because of the mistaken assumption that microscopic size is synonymous with simplicity. Through this error, investigators have failed to recognize, and hence, to limit the number of unknown factors involved in their work, a fact which is well exemplified by the numerous methods employed in producing bacterial vaccines. Gross injections of bacterial cultures, of one or many strains, killed or attenuated by heat, chemicals, or other lethal agents, or extracted and triturated by such solvents as suggest themselves, have been used with the hope that somehow enough of the substances that cause antibody formation might be obtained to produce the desired result. From what we know of the sensitiveness of proteins to such radical treatment, the results have been far more satisfactory than could well have been expected.

With regard to bacterial antigens, a series of investigations by numerous investigators whose works cover a period of years, has brought out the facts that the most potent antigen is obtained (1) from autolysed bacteria rather than from whole bacterial bodies, and (2) from bacterial proteins which are least altered from the natural state in the living protoplasm. (Altered proteins, at best, retain only the basic structures which are capable of reacting immunologically, to give only the broader group specificity.) Thus Gay and Claypole [1] in their study of bacterial vaccines, conclude that the living culture, or its fresh unaltered autolysate, is the most effective; but they precipitated the organisms from saline suspensions by adding an equal volume of absolute alcohol, then dried them over sulphuric acid and finally ground them with crystals of sodium chloride as an abrasive. Hahn used the Buchner press to obtain the extract. Wassermann autolysed cultures with dis-

Received for publication, Dec. 4, 1925.

[1] Arch. Int. Med., 1924, 14, p. 671.

tilled water. Still others have used ball mills. In all of these methods, due to the slowness or incompleteness of the processes, there is danger of denaturing because of bacterial contamination or for other reasons.

In a long series of experiments on the antigenic properties of the proteins of seeds of certain flaxes,[2] it has been shown that the globulin fractions are highly specific as antigens whereas the other protein fractions are nonspecific. They have found that this specificity is distinct enough to distinguish between one selection variety of plants which is resistant to flax wilt, and another selection, nonresistant. But this specificity is lost sight of in the conglomerated proteins of the flax seeds.

In view of these results, it seemed desirable to test the antigenic properties of the globulin fractions from autolysed bacteria, since globulins, if found, represent simpler protein complexes than the mixtures usually used. As far as has been ascertained no definite experimental work on the determination of globulins in bacteria has been recorded. The researches of Wells,[3] Osborne,[4] Wodehouse,[5] and others on the proteins of plants, however, offer a basis for the application of improved methods in this study.

The purpose of the present work is to give a method of obtaining purified globulin from bacteria for use as antigen, and to determine their value as specific antigens. If such bacterial globulins, used as antigens, give a narrow specificity, they should offer (1) a means to study group and phylogenetic relationships among bacteria, (2) a means to study "endotoxin" variation, and (3) serve to improve present diagnostic methods.

EXPERIMENTAL WORK

Seven members of the colon-typhoid group of bacilli, representative of the various main sub-groups, viz., B. coli-communis, B. paratyphosus A, B. paratyphosus B, B. typhosus (Hopkins strain), B. enteritidis, B. dysenteriae Flexner and B. dysenteriae Shiga, were cultured en masse on a specially devised nonprotein medium. Because of the difficulty of obtaining a sufficient mass of bacteria with a liquid medium, agar was added as the most efficient agglutinant. In view of the fact that the globulin fractions obtained in the present work are to be used later for antigenic purposes, it was considered essential that no extraneous protein be used in the medium. Melick has shown that traces of protein

[2] Nelson and Dworak: Unpublished manuscript, N. Dakota Agric. Exper. Sta.

[3] J. Infect. Dis., 1909, 6, p. 506; 1911, 8, p. 66; 1913, 12, p. 341; 1915, 17, p. 259; 1916, 19, p. 183.

[4] Proc. Soc. Exper. Biol. & Med., 1908, 5, p. 105; Am. Chem. J., 1892, 14, p. 629.

[5] Boston M. & S. J., 1916, 175, p. 195.

from the medium carried over with the bacterial mass into the autolysate, enter into the sensitization of animals, and so produce confusing results. Consequently, to eliminate the foreign protein débris found in agar,[6] the shreds were leached 48 hours in numerous changes of distilled water. The bacterial cells were also thoroughly washed.

The following medium possesses the advantages of being simple, readily prepared, easily sterilized in the autoclave at 10 pounds pressure for 20 minutes, and has a final unadjusted reaction of P_H 7.0 to 7.2 to brom-thymol-blue:

Distilled water	1000 cc.
Dibasic potassium phosphate	2 Gm.
Ammonium succinate	2 Gm.
Sodium chloride	2 Gm.
Dextrose	2 Gm.
Agar shreds	20 Gm.

All cultures were grown in Kolle flasks for from 48 to 72 hours at 37 C. They were then washed off with sterile isotonic saline solution and repeatedly thrown out of suspension from fresh saline solution until the supernatant fluid gave negative tests for protein. About 2 cc. of a pasty bacterial mass was obtained from 10 Kolle flask cultures of the faster growing organisms.

Autolysis.—This bacterial mass was subsequently resuspended in 7 cc. isotonic saline solution and subjected for 10 consecutive times to alternate freezing in liquid air and thawing in hot water as a means of autolysing the cells.[7] The resulting fluid had the consistency of a thin solution of egg white. Microscopic examination of the cellular débris left from centrifuging the autolysate showed that from 95% to 98% of the bacterial cells were vacuolated and had lost all but a thin layer of material next to the cell wall. The extent of autolysis was readily checked by this means. It was noted that some cultures autolyzed more readily than others. From 6 to 8 alternate freezings and thawings autolyzed all of the cultures except B. coli as thoroughly as 10 such treatments did the latter. The clarified autolysates gave definite biuret and other protein color tests.[8] In reaction it ranged from P_H 5.8 to 6.0.

[6] Fellers, C. R.: J. Indust. & Engin. Chem., 1916, 8, p. 1128.

[7] Macfadyen and Rolands: Centralbl. f. Bakteriol. I. O., 1903, 34, pp. 618, 765. Melick, C. O.: J. M. Res., 1922, 43, p. 405.

[8] Since only very small amounts of material could be spared for testing, excellent results were obtained by performing the protein tests in very small precipitin tubes, applying the reagents with fine capillary pipets.

Fractionation by Ammonium Sulphate.—When the autolysates were half saturated with ammonium sulphate a heavy precipitate, protein in nature, formed within 15 minutes. If removed from the supernatant fluid at once, the precipitate redissolved readily in 1% sodium chloride solution. On longer standing resolution became more difficult. Dialyzed in collodion sacks or parchment paper bags against running tap-water, and then against standing distilled water, it reprecipitated. About 10 days were required for precipitation by this method, and the yield was low.

Further saturation of the remaining solution (to 75% saturation by weight with ammonium sulphate) yielded a very small amount of a dirty gray precipitate, doubtfully protein, and only slightly soluble in 5% sodium chloride solution. Acidification and complete saturation of the remaining fluid gave no evidence of albumin.

Fractionation by Electrodialysis.—The method of electrodialysis suggested by Locke and Hirsch [9] offers a means of rapidly dialyzing and purifying proteins. It avoids the danger of denaturing attendant on salting out by ammonium sulphate. Likewise, it provides a condition under which micro-organisms that thrive at the expense of the material being dialyzed by the old method, cannot grow.

Two dialyzing thimbles containing platinum electrodes or electrodes of carbon and provided with inlet and outlet for circulating water, were placed in a vessel containing the bacterial autolysate, unaltered by the addition of any salts. A direct current, fitted with such resistance that there was a potential drop to about 45 volts between the electrodes, was permitted to act continuously for 8 to 12 hours. Even with a reading of 110 volts there was no perceptible heating of the liquid system when a steady supply of cold tap water was passing through the thimbles. The water which circulates through the dialyzing thimbles serves to keep the units cooled below the temperature at which the proteins may be altered and may, if necessary, be adjusted in reaction to approach their iso-electric point. Water from the laboratory tap was used since its P_H favored the approach to the iso-electric point of the bacterial globulins. In another locality, with a water supply high in alkaline salts (Fargo, N. D.), it was found necessary to use distilled water for this purpose.

The precipitation of the globulin took place in from 6 to 8 hours. A zone of clear fluid was formed over the layer of precipitate. This clear

[9] J. Infect. Dis., 1924, 35, p. 519.

TABLE 1

RESULTS OF PRECIPITIN TESTS WITH BACTERIAL GLOBULINS

1. B. typhosus antiserum

Globulin	Serum Dilutions 1:10	1:20	1:40	1:80	1:100	Control
B. typhosus						
Test 1 (17 hrs.)	0	+++	+++	T	?	0
Test 2 (4 hrs.)	++++	++++	++	++	T	0
B. paratyphosus A	+++	+	T	T	T	
B. dysenteriae Flexner						
Test 1	?	?	+	0	0	0
Test 2	+	T	T	?	0	0

2. B. dysenteriae Flexner antiserum

Globulin	Serum Dilutions 1:10	1:20	1:40	1:80	1:100	Control
B. dysenteriae Flexner						
Test 1 (24 hrs.)	+++	++	0	0	0	0
Test 2 (4 hrs.)	++++	++	+	T	0	0

3. B. paratyphosus A antiserum

Globulin	Serum Dilutions 1:10	1:20	1:40	1:80	1:100	Control
B. paratyphosus A						
Test 1 (17 hrs.)	++	++	+	0	0	0
Test 2 (4 hrs.)	++++	+++	+	0	0	0
B. typhosus	—	—	T	T	0	0

4. B. coli antiserum

Globulin	Serum Dilutions 1:10	1:20	1:40	1:80	1:100	1:200	Control
B. coli							
Test 1 (17 hrs.)	++++	+++	+++	+	T	0	0
Test 2 (4 hrs.)	++++	++++	+++	++	T	0	0

5. B. enteritidis antiserum

Globulin	Serum Dilutions 1:10	1:20	1:40	1:80	1:100	Control
B. enteritidis	++	++	+	T	0	0
B. paratyphosus	+	T	T	0	0	0
B. dysenteriae Flexner	+++	++	+	T	T	0
B. typhosus	?	++	+	+	+	
B. coli	++	+	T	0	T	0

fluid was without trace of protein when tested by color reactions, salting out with ammonium sulphate, or acidifying and heating. It was evident that electro-dialysis removes practically all of the globulin. The globulin obtained by this method was insoluble in distilled water but dissolved readily in dilute salt solution. When redialyzed it precipitated again in a very few hours, and in more compact form. It was positive to the biuret and other color tests for protein. Although electrodialysis deposits a layer of pseudoglobulin over the euglobulin when blood serum is dialyzed there was no evidence of such layering with bacterial autolysates.

Antigenic Properties of Purified Bacterial Globulin.—Only small amounts of globulins were obtained from the bacterial masses by autolysis and electrodialysis. These were dissolved in 25 cc. of sterile physiological salt solution for injection as antigen. Half grown rabbits were sensitized to the globulins from B. typhosus, B. paratyphosus A, B. dysenteriae Flexner, B. enteritidis, and B. coli communis, by several intraperitoneal injections spaced 3 or 4 days apart. Five days after the last injection 15 cc. of blood was drawn from the ears of the animals and the serum tested for precipitins against the homologous antigens.

In previous work [2] in which only minute amounts of the antigen were available, tests were performed keeping the concentration of the antigen constant and varying only the serum dilutions. The same method was followed in this work. The globulin solutions used as antigens were centrifuged at 800 to 1,000 r.p.m. for 5 minutes. Some of this material was kept in tubes in the refrigerator as a check on the possibility of change, in the solubility of the antigen and its consequent sedimentation. Following the addition of the serum to the antigen the tubes were agitated to obtain a uniform mixture of the two fluids. By this means the ring formation which occurred at the interface of the antigen and the serum in some of the higher concentrations disappeared. All tubes were then incubated in a Wassermann serum bath at 37 until a more or less compact flocculum formed. When this occurred within 18 hours it was considered a positive precipitin reaction.

In the following data the estimation of the amount of flocculum is indicated by one or more plus signs, or the letter T (trace). It becomes an estimation of the precipitin titer. Specificity is put on a quantitative basis by a comparison of the quantities and ranges of precipitates formed with the antigens and the homologous or heterologous serums.

DISCUSSION

Titration of the globulin antigens with normal rabbit serum indicated that in normal rabbit serum there were no natural precipitins for these bacterial globulins. In all immune serums the homologous antiserums and antigens reacted strongly. The antiserums produced by 3 injections of the animals showed precipitin in a dilution of 1 : 40. Antiserums of higher potency were produced by more injections. This is indicated both by an extension of the effective range of serum dilution to 1 : 100 and a reduction of the time necessary for the formation of the flocculum.

A study of the cross titrations with precipitin produced against B. typhosus globulin shows that though it reacts to the homologous antigen within a range of 1/80, it is sensitive to the globulin of a closely related organism, B. paratyphosus A, within a range of only 1/20, and with that of a more distantly related organism, B. dysenteriae Flexner, within a narrow range of 1/10. An inversion of the elements tested (table 1, part 3) does not indicate the same close relationship between B. paratyphosus A and B. typhosus. The data in part 5 might indicate close phylogenetic relationship except for the possibility as suggested in part 3, that an inversion of the elements of the test might not give data in agreement with this. A better understanding of the significance of the results of cross tritration will be possible when more exhaustive studies have been made. A narrow specificity of these bacterial globulins for their homologous precipitin is plainly indicated by the data given. It remains to be seen whether these bacterial globulins when titrated against antisera produced with whole bacteria, will be of diagnostic value in distinguishing between closely related members of the same group.

SUMMARY

A synthetic medium suitable for culturing members of the colon-typhoid group of bacteria en masse is described.

A method of obtaining purified bacterial globulins by autolysis and electrodialysis is suggested. The time required for obtaining these purified globulins by this method may be less than 24 hours.

Globulins obtained from B. typhosus Hopkins, B. paratyphosus A, B. dysenteriae Flexner, B. enteritidis and B. coli serve as highly specific antigens for the production of precipitins.

The specificity of these globulins for their homologous precipitins is measured on a comparative, quantitative basis.

Data offered suggest this method of precipitin testing as a means to study group relationships between closely related bacterial species.

ARE THERE IMMUNOLOGIC STRAINS OF SPIROCHAETA PALLIDA?

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The theory of specificity or "selective affinity" of strains of spirochaeta pallida is largely based on clinical observations, particularly of the central nervous system. From the experimental standpoint the original observations of Nichols and Greene and Reasoner bearing upon the production of eye lesions in rabbits, followed later by the work of Levaditi and his colleagues on so-called "neurotropic" and "dermatropic" strains, has yielded some support to the theory of specific strains, but it is not at all improbable that Spirochaeta cuniculi may have confused the results of some of the experiments abroad. At least too little attention has been paid to the influence of virulence of strains, to trauma and other constitutional differences relating to the host, and to the conditions under which infection was contracted as factors capable of influencing the localization of Spirochaeta pallida and clinical variations in the course of syphilis.

We have approached the solution of this problem in a different manner from that hitherto published. By cross agglutination and complement fixation tests with the serums of rabbits immunized with different strains of Spirochaeta pallida we have tested the demonstrability of different immunologic strains analogous to the fixed serologic types of pneumococci and meningococci.

Six strains of Spirochaeta pallida were cultivated in vitro and sufficient organisms obtained for the immunization of rabbits and for a complete set of complement fixation and a few agglutination tests. These strains were designated as follows:

(*a*) Nichols strain, being the original strain secured by Nichols and Hough in 1912 from the cerebrospinal fluid of a case of neurosyphilis and since widely employed for the production of experimental infections of rabbits.

(*b*) Rule (R.S.) strain isolated from a mucous patch.

(*c*) Rule (G.D.) strain isolated from a chancre.

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(*d*) Noguchi strain designated as (C); one of Noguchi's original strains but source unknown.

(*e*) Weiss (H) strain isolated from a vulvar chancre.

(*f*) Greenbaum (G.A.) strain isolated from a chancre.

All of these strains except that of Weiss (H) had been passed through numerous rabbits before cultivation in vitro; the Weiss (H) strain was successfully cultivated direct from the lesion. We are certain that none of our strains was contaminated with Spirochaeta cuniculi of rabbit spirochetosis. Unfortunately, however, it was possible to use only one strain from the central nervous system, namely, the Nichols-Hough strain.

Rabbits were given from 7 to 14 intravenous injections of heat killed cultures or saline suspensions of the different strains and bled about 7 days after the last injection. Preliminary agglutination tests showed that the serum of a rabbit may cause slight agglutination in final dilution of 1 : 10, but following immunization the agglutinin titers varied from 1 : 300 to approximately 1 : 600. These tests were conducted by mixing in small test tubes 0.5 cc. of varying dilutions of serum with 0.5 cc. of spirochete suspensions and incubating two hours at 37 C. The tubes, along with controls, were then placed in a refrigerator over night and the contents of each examined next day by dark field microscopy.

The complement-fixation tests were conducted according to the technic of the new method developed by one of us (J.A.K) for the serum diagnosis of syphilis. Each antigen was employed in a dose equal to one third its anticomplementary unit with varying amounts of serum, namely, 0.05, 0.01, 0.005 and 0.0025 cc. previously heated at 60 C. for 30 minutes. These amounts of rabbit serum do not yield the non specific complement fixation reactions in this method which are so commonly observed with different antigens. Primary incubation was 18 hours at 6 to 8 C. followed by 10 minutes in a water bath at 38 C.

Each immune serum was tested against the 6 different strain antigens for complement fixation and the results are summarized in table 1. The antibody content of the respective serums varied somewhat as would be expected but we were unable to observe that any serum possessed unusual or specific complement-fixing properties for its own or homologous antigen. In other words, the results of complement-fixation tests did not indicate immunologic specificity on the part of any of the strains of spirochaeta pallida employed in this investigation.

Unfortunately, we were not able to prepare sufficient antigens of the different strains for the conduct of a complete series of cross agglutination tests because of the unsurmontable difficulties of inducing several of the strains to grow sufficiently well in a fluid medium for producing suspensions of sufficient density. But the results of one experiment shown in table 2, are representative of the whole and like the complement-fixation tests have failed to demonstrate immunologic specificity on the part of the strains employed in our work.

TABLE 1

Complement-Fixation Reactions

Strains (antigens)	Serum (Nichols)			Serum (R. S.)			Serum (G. D.)		Serum (C)		Serum (H)			
	0.025	0.01	0.005	0.025	0.01	0.025	0.025	0.01	0.025	0.01	0.025	0.01	0.005	0.0025
Nichols	+++	++	0	+++	++	0	++	0	++	0	++++	+++	+	0
Rule (R. S.)	++	0	0	+	0	0	+	0	+	0	++	0	0	0
Rule (G. D.)	++	0	0	++	+	0	+	0	+	0	++	+	0	0
Noguchi (C)	++	0	0	++	0	0	++	0	+	0	+	0	0	0
Weiss (C)	++++	+++	0	0	0	0	+	0	++	0	++++	++	0	0
Greenbaum (G. A.)	++++	+	0	+	0	0	+	0	+	0	++	+	0	0

TABLE 2

Agglutination Tests with the Serum of Rabbit Immunized to the Noguchi Strain of Spirochaeta Pallida

Strains	Highest Active Dilution of Serum
Nichols	640
Rule (R. S.)	640
Rule (G. D.)	160
Noguchi (C)	640
Weiss (H)	160
Greenbaum (G. A.)	320

SUMMARY

Possibly the study of a larger number of strains by similar methods may show the existence of immunologically distinct types of Spirochaeta pallida, but no evidence of the existence of such was obtained in our investigation.

On the contrary our results may lend some confirmation to the view that in so far as experimental syphilis of the rabbit is concerned, the localization of Spirochaeta pallida and the subsequent course of the disease are largely influenced by the virulence of the organism and method of inoculation as well as by the susceptibility of the host and the efficiency of its defensive reaction. Probably the same or similar factors are operative in syphilis of human beings without involving the question of strain specificity or "selective tissue affinity" of the infecting spirochetes.

HEMOLYTIC STREPTOCOCCI OF THE BETA TYPE IN CERTIFIED MILK

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In a paper read at the annual session of the American Medical Association at Atlantic City, N. J., in May, 1925, Bundesen [1] described the following method as having been adopted by the Chicago Health Department for the safeguarding of raw certified milk from streptococci which may be pathogenic for man:

> Hemolytic organisms found in certified milk should be regarded as of no significance unless conforming to the following characteristics:
>
> They should be streptococci of proper morphology, giving colonies of the beta type on blood agar.
>
> Production of hemolysin should be sufficient to produce full hemolysis of rabbits' blood corpuscles within two hours.
>
> They should kill both of two mice injected, acutely within forty-eight hours, and should be recovered from the animals and identified.
>
> Samples containing organisms conforming to these tests may be regarded as under suspicion, but these data are merely suggestive and not proof that the organism is dangerous to human beings. Any action which may be taken must necessarily be determined by practical circumstances. If, however, an absolute safeguard against the possibility of throat outbreaks is desired, pasteurization or a shut-off of the supply until subsequent samples prove negative is the only reliable procedure.

Bundesen points out that "the question of the infectiveness of milk containing streptococci has not been definitely determined" and that there are still "problems that must be solved in connection with the safeguarding of this product." The group of streptococci collectively known as Streptococcus hemolyticus is a large one including streptococci of different origin and cultural characteristics, many of them harmless to man and to be found frequently in the best grades of raw and pasteurized milk. The task confronting the health department which attempts to prevent the occurrence of streptococcus epidemics is much more difficult than the tracing of such an epidemic to its source. The former problem must be attacked by the bacteriologist without the help of the epidemiologist.

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[1] J. Am. M. Assn., 1925, 85, p. 1285.

In accordance with the above procedure the Chicago Health Department in the spring of 1925 had isolated from certified milk a number of strains of streptococci regarded as suspicious although no epidemic among the consumers of the milk was reported. These results were reported to the Medical Milk Commission of the Chicago Medical Society and, since a large percentage of the certified milk used in Chicago is produced on farms in Wisconsin, the Medical Milk Commission arranged for an investigation of the problem to be made by the University of Wisconsin. This paper is the initial report of this investigation carried out during the months of July and August, and embraces a study of all the beta hemolytic streptococci which could be isolated from five certified herds including about 900 cows producing milk for the Chicago market, and a comparison of these streptococci with those isolated by the Chicago Health Department and with strains previously isolated from epidemics of septic sore throat.

Smith and Brown [2] concluded that "the streptococci causing epidemics of tonsillitis are not necessarily the same in different epidemics either in the same or different localities." In only one of the epidemics (Canton, Mass.) reported by Smith and Brown can it be said that the same streptococcus found in the patients was also isolated from the suspected milk. The other epidemics were not proven bacteriologically to be of milk-borne origin. However, there are now on record a number of epidemics of septic sore throat in which the epidemiological data indicated that the infection was milk-borne, and, in which, streptococci from the patients were apparently identical with strains isolated from the suspected milk and in most cases from the milk of a single cow. The rare occurrence of such streptococci in the bovine udder led Savage,[3] and Smith and Brown, independently in 1915, to advance the theory that such epidemics are not caused by the streptococci which usually cause bovine mastitis but by streptococci of human origin which occasionally gain entrance to the udder of the cow. Davis and Capps [4] succeeded in infecting the cow's udder with human streptococci by rubbing the culture on the abraded teat. Savage [3] did not find any constant cultural difference between the streptococci of human and bovine origin, but regarded the pathogenicity for mice as of considerable significance. Injected subcutaneously the bovine streptococci were of low virulence and frequently nonpathogenic for mice. Human strains frequently

[2] J. M. Research, 1915, 31, p. 455.
[3] J. Meat & Milk Hyg., 1911, 1, p. 175.
[4] J. Infect. Dis., 1914, 15, p. 135.

killed mice. He is careful to state, however, that the distinction is one of degree rather than of kind. Salter[5] found most beta hemolytic human streptococci pathogenic for mice when injected intraperitoneally whereas hemolytic strains from pasteurized milk were nonpathogenic, but strains from high grade raw milk frequently caused the mice to be sick and might produce death. The milk was not under suspicion, and no cases of infection among the consumers were reported.

Since 1914 new aids for the differentiation of streptococci of human and bovine sources have come into use. Chief among these are (1) the recognition of different types of streptococci in blood agar,[2, 6] (2) the hemolytic titer in fluid media,[7] (3) the hydrogen ion concentration attained in glucose broth[8] and (4) the hydrolysis of sodium hippurate.[9]

The results of the present study are summarized in table 1. Group 1 includes the strains which we regard as of human type, i. e., those capable of producing disease in human beings. Of those designated Streptococcus epidemicus most are from human cases in the epidemics indicated under "Source"; a few are from the milk of cows incriminated in these epidemics, and three strains (Blackie, Beauty and Mkt. 1) are from milk without history of having caused human infection. Two of these are from gargety cows on a small isolated poorly kept farm and the milk was not used for human consumption. The three strains of Streptococcus pyogenes are from sporadic cases of sore throat, and the two strains of Streptococcus infrequens are from sporadic cases of septicemia and pneumonia respectively. Strain 8003 is from a septicemia. These six strains are tabulated merely for comparison with the epidemic strains and with certain of the bovine strains which have similar fermentation reactions. The strains of group 1 have certain characteristics in common. (1) They hemolyze rabbit blood corpuscles suspended in fluid media in less than two hours. (2) With the exception of strain 8003 they are markedly pathogenic for mice; 0.5 cc. injected intraperitoneally usually causing death in a few hours. (3) In 1% glucose broth they produce a final hydrogen ion concentration not higher than P_H 4.8. (4) All but strain 8003 ferment salicin. (5) They do not hydrolyze sodium hippurate. The strains of Streptococcus epidemicus form a homogeneous sub-group. They ferment glucose, lactose, saccharose and salicin but not mannite and are similar to Strepto-

[7] U. S. Medical War Manual No. 6. 1919. Brown, J. H.: J. Exper. Med., 1920, 31, p. 35.

[5] Am. J. Hyg., 1921, 1, p. 154.

[6] Brown, J. H.: Monograph No. 9, Rockefeller Inst. for Medical Research, 1919.

[8] Ayers, S. H.: J. Bact., 1916, 1, p. 84. Ayers, Johnson and Davis: J. Infect. Dis., 1918, 23, p. 290. Avery and Cullen: J. Exper. Med., 1919, 29, p. 215.

[9] Ayers and Rupp: J. Infect. Dis., 1922, 30, p. 388.

coccus pyogenes in all respects except that they possess a well defined capsule when observed in a moist india ink preparation. The possession of capsule is often accompanied by a characteristic mucoid confluent or amoeboid character of the young colony on fresh blood agar as distinguished from the round discrete colony of the nonencapsulated Streptococcus pyogenes. The name Streptococcus epidemicus was first used by Davis;[10] the first adequate descriptions were given by Davis and Rosenow,[11] and more completely by Davis.[12] For our present purpose it makes little difference whether this form of streptococcus is regarded as a distinct species or as a variety of Streptococcus pyogenes. It may be that capsules may be acquired by Streptococcus pyogenes under favorable conditions, (in fact we have been able to induce capsule formation in the laboratory), but if so these conditions appear to exist

TABLE 1

The Differential Characters of Beta Hemolytic Streptococci from Milk, from Septic Sore Throat and from Sporadic Human Infections

Group	Source	Number	Capsule	Hemolytic Titer	Pathogenicity for Mice	Fermentation: Glucose	Fermentation: Lactose	Fermentation: Sucrose	Fermentation: Mannite	Fermentation: Salicin	Hippurate Hydrolysis: FeCl3 Test	Hippurate Hydrolysis: Formol Titer
1	Streptococcus epidemicus											
	Boston '11	X-32	+	++++	+ 6 days	5.2	+	+	0	+	0	.....
	Baltimore '12	X-39	+	++++	+ 2 days	5.2	+	+	0	+	0	—0.05
	Chicago '12	X-40	++	+++	+ 9 hours	5.2	+	+	0	+	0	—0.1
	Canton '13	LeF	+	++++	+ 19 hours	5.9	+	+	0	+	0	.....
	Canton '13 cow	B-2b	+	+++	+ 19 hours	5.2	+	+	0	+	0	.....
	Jacksonville '14	*	+	.....	.....	+	+	+	0	..	..	.....
	Boston '17	H-M	++	+++	+ 9 hours	5.2	+	+	0	+	0	—0.4
	Boston '17 cow	H-108	++	++++	+ 19 hours	5.2	+	+	0	+	0	.....
	Galesville '17	*	+	.....	.....	+	+	+	0	..	..	.....
	Saugetiers '25	M-2706	++	++++	+ 20 hours	5.3	+	+	0	+	0	.....
	Saugetiers '25	M-2795	+	++++	+ 23 hours	5.1	+	+	0	+	0	.....
	Cow '25	Blackie	++	++++	+ 21 hours	4.8	+	+	0	+	0	.....
	Cow '25	Beauty	++	++++	+ 21 hours	4.9	+	+	0	+	0	—0.15
	Market milk	Mkt. 1	+	+++	+ 48 hours	5.4	+	+	0	+	0	.....
	Streptococcus pyogenes											
	Throat	Pv-8	0	++++	+ 15 hours	5.1	+	+	0	+	0	—0.2
	Throat	P-23	0	++++	+ 4 days	5.4	+	+	0	+	0	.....
		C.B.H.-1	0	+++	+ 8 hours	5.1	+	+	0	+	0	—0.1
	Streptococcus infrequens											
	Necropsy	8596	0	++++	+ 19 hours	4.9	+	+	+	+	0	—0.3
	Necropsy	8606	0	++++	+ 8 hours	4.9	+	+	+	+	0	—0.15
	Streptococcus anginosus											
	Necropsy	8003	0	+++	0	5.1	+	+	0	0	+	.....
2	Certified milk	1	0	++	0	4.4	+	+	0	+	++	.....
	Certified milk	2	0	++++	+ 6 days	4.5	+	+	0	+	++	.....
	Certified milk	3	0	+++	0	4.5	+	+	0	+	++	.....
	Certified milk	4	0	++	.....	4.5	+	+	0	+	++	.....
	Certified milk	5	0	++++	.....	4.6	+	+	0	+	++	.....
	Certified milk	6	0	+++	+ 4 days	4.5	+	+	0	+	0	0.2
	Certified milk	7	0	++	+ 60 hours	4.5	+	+	0	+	++	4.5
	Certified milk	8	0	++	.....	4.5	+	+	0	+	++	.....

[10] J. Am. M. Assn., 1912, 58, p. 1283.

[11] Ibid., p. 773.

[12] Ibid., p. 1853.

TABLE 1—Continued

Group	Source	Number	Capsule	Hemolytic Titer	Pathogenicity for Mice	Fermentation: Glucose	Fermentation: Lactose	Fermentation: Sucrose	Fermentation: Mannite	Fermentation: Salicin	Hippurate Hydrolysis: $FeCl_3$ Test	Hippurate Hydrolysis: Formol Titer
3	Certified milk	9	0	++++	.....	4.7	+	+	+	+	0	.....
	Certified milk	10	0	++++	0	4.5	+	+	+	+	++	4.6
	Certified milk	C-1392	0	++++	+ †	4.6	+	+	+	+	±	1.3
	Certified milk	C-1393	0	.....	+ †	4.7	+	+	+	+	±	1.4
	Certified milk	C-2081	0	++++	+ †	4.8	+	+	+	+	sl.	1.15
	Certified milk	C-2082	0	++++	+ †	4.7	+	+	+	+	sl.	1.3
	Certified milk	C-2735	0	++++	+ †	4.7	+	+	+	+	0	0.95
	Certified milk	C-2083	0	.....	+ †	4.7	+	+	+	+	sl.	0.75
	Certified milk	C-2858	0	++++	+ †	4.7	+	+	+	+	±	1.25
	Certified milk	C-3052	0	++++	+ †	4.7	+	+	+	+	sl.	0.95
	Certified milk	C-3056	0	++++	+ †	4.7	+	+	+	+	0	1.05
	Certified milk	11	..	++++	0	4.7	0	+	+	+	+	1.2
	Certified milk	12	..	++++	0	4.6	+	+	+	+	+	1.1
	Certified milk	13	0	++++	+ 13 days	4.7	+	+	+	+	0	.....
	Certified milk	14	0	++++	+ 6 hours	4.8	+	+	+	+	0	.....
4	Certified milk	15	0	++++	+ 22 hours	4.5	+	+	0	0	++	.....
	Certified milk	C-2569	0	++++	+ †	4.7	+	+	0	0	++	5.05
	Certified milk	C-2667	0	++++	+ †	4.7	+	+	0	0	++	.....
	Certified milk	16	..	++++	0	4.8	+	+	0	0	++	4.7
	Certified milk	17	0	++++	+ 4 hours	5.1	+	+	0	0	++	4.4
5	Certified milk	18	0	++++	.....	4.7	+	6.0	0	0	++	.....
	Certified milk	19	0	++++	+ 7 hours	4.8	+	6.2	0	0	++	.....
	Certified milk	20	±	++++	0	4.7	+	6.5	0	0	0	0.05
6	Certified milk	21	sl.	++++	0	4.7	+	0	0	0	0	0.2
7	Certified milk	22 to 76	0	0	±	4.4 or 4.5	+	+	0	+	++	2.05 to 4.75 (8 strains)
	Of the 55 strains in this group a few produced slight hemolysis in test tubes; of 9 strains injected into mice 4 were non-pathogenic, 3 killed mice within 48 hours and 2 within 7 days. One strain produced a slight capsule.											
8	Certified milk	77	0	+	0	4.7	+	+	+	+	0	.....
	Certified milk	78	0	0	.....	4.5	+	+	+	+	++	4.64
9	Certified milk	79	0	0	.....	4.5	+	+	0	0	++	.....
	Certified milk	80	0	0	.....	4.5	+	+	0	0	++	.....
	Certified milk	81	0	0	.....	4.5	+	+	0	0	++	.....
	Certified milk	82	0	0	.....	4.5	+	+	0	0	++	.....
	Certified milk	83	..	sl.	.....	4.6	+	+	0	0	++	.....

The figures given under fermentation represent the final P_H as determined by the method of Brown.[13]

Many of the above strains were tested for fermentation of raffinose. None fermented this substance.

Formal titrations were done by the method of Brown.[14] The figures are in terms of % normality but may be reduced to mg. of nitrogen per 100 cc. of culture by multiplying by 14. The results express the increase in formol titratable substances over those titrated in sterile controls of the same media. In group 1 the figures preceded by a — sign indicate that less formol titratable substance was found in the cultures than in the controls.

± means doubtful; sl. means slight.

* We did not have an opportunity to study the Jacksonville and Galesville strains. The results tabulated are taken from the publications of Capps and Davis[15] and Rosenow and Hess[16] respectively.

† Results of mouse inoculations reported to us by the Chicago Health Department.

[13] J. Lab. and Clin. Med., 1924, 9, p. 239.

[14] J. Bact., 1923, 8, p. 245.

[15] Arch. Int. Med., 1914, 14, p. 650.

[16] J. Am. M. Assn., 1917, 68, p. 1305.

in connection with milk-borne epidemics of septic sore throat. Furthermore, the strains which we have kept from such epidemics have persisted in forming capsules throughout years of cultivation in the laboratory. This then is the type of streptococcus which should be sought for as the actual or potential cause of epidemics of septic sore throat—a streptococcus of round or slightly flattened elements, producing hemolyzed zones of the beta type in blood agar, rapidly hemolyzing blood corpuscles in fluid media, markedly pathogenic for mice, fermenting glucose, lactose, saccharose and salicin but not mannite, producing a final hydrogen ion concentration not above P_H 4.8 in glucose broth, failing to hydrolyze sodium hippurate, and probably always encapsulated when observed under suitable conditions, in the last respect differing from most strains of hemolytic streptococci isolated from sporadic human infections.

The strains of groups 2 to 9 inclusive in table 1 are beta hemolytic streptococci from certified milk. Those with the letter C as prefix were isolated by the Chicago Health Department and regarded as suspicious because of their hemolytic activity and pathogenesis for mice. In this investigation it has been our pleasure to enjoy the cooperation of the Chicago Health Department, the Medical Milk Commission of Chicago and the Certified Milk Producers. Of the strains numbered 1 to 83 seventeen were from individual cows in certified herds. Most of these 17 cows were gargety and the milk was excluded from the supply by the producers. These streptococci differed in no respect from similar strains isolated from clinically normal cows and are tabulated throughout groups 2, 3, 4, 5, 7, 8 and 9. The remaining strains from certified milk were isolated from samples of the mixed milk of groups of about ten cows in the milking herds. With two or three rather doubtful exceptions none of the streptococci from these animals or from the group samples of mixed milk were encapsulated, and these strains differed from Streptococcus epidemicus in other respects. The strains of groups 2 to 6 inclusive produced marked hemolysis of blood corpuscles in fluid media within two hours. Not all of the strains were injected into mice but strains pathogenic for mice are to be found scattered throughout most of the tabulated groups. The kind of medium in which the organisms were grown exerted a marked effect on pathogenicity for mice; plain infusion broth cultures often failing to kill mice whereas serum broth cultures killed them within a few hours. The strains of groups 2 and 7 fermented the same substances as did Streptococcus epidemicus but differed from the latter in producing a higher acidity (P_H 4.5) and actively hydrolyzing sodium

hippurate, as did also the strains of group 9. Groups 3, 4 and 5 contain a few strains which produce the same hydrogen ion concentration as the human strains in group 1 but those of groups 4 and 5 failed to ferment salicin and all but one (No. 20) hydrolyzed sodium hippurate. The strains of group 3 including most of those isolated by the Chicago Health Department differed from the human strains in fermenting mannite. In their final hydrogen ion concentration and in their action on sodium hippurate they occupy an intermediate position between the other bovine strains and the human strains. Their final hydrogen ion concentration in glucose broth was about P_H 4.7 and formol titrations showed an appreciable hydrolysis of sodium hippurate. They form an interesting group hitherto undescribed. On the basis of hemolytic activity and pathogenesis for mice they might well be regarded as suspicious but they are not the type found in epidemics of septic sore throat in man. They failed to cross agglutinate with strains of Streptococcus infrequens (8596 and 8606) isolated from human necropsies.

A more complete serological study of the various groups of streptococci is reserved for a later report.

CONCLUSION

Beta hemolytic streptococci in considerable numbers were found in the cretified milk of five dairy herds. These streptococci belonged to several distinct cultural and serological groups. There is no evidence that any of them, whether from gargety cows or from mixed milk, are pathogenic for human beings. The causative organism of milk-borne septic sore throat is Streptococcus epidemicus. This streptococcus is probably of human origin but occasionally may gain entrance to the udder of a cow and find its way into the milk supply. The hemolytic streptococci commonly found in milk show distinct cultural and biological differences from Streptococcus epidemicus. No single test, however, can be relied upon to differentiate them. All of the tests listed in table 1 are of value. A few bovine strains may possess capsules. Many produce rapid hemolysis of blood cells in fluid media. A considerable number are markedly pathogenic for mice. Many ferment the same sugars as the human strains. A few produce about the same final hydrogen ion concentration in glucose broth as do the human strains and a few resemble the latter in producing little or no hydrolysis of sodium hippurate. The latter two tests, however, are probably the most constant and reliable of those tried.

A routine procedure for the detection of Streptococcus epidemicus in milk may be outlined as follows:

1. Milk should be inoculated into poured blood agar plates.

2. Capsules should be looked for in moist india ink preparations from young moist surface colonies.

3. Deep colonies with beta zones of hemolysis are to be fished into serum broth. To save time fishings from the same colonies may be made into glucose broth and into sodium hippurate broth.

4. From the broth culture make: (*a*) A hemolysin test, adding 0.5 cc. of a 5% suspension of washed rabbit blood corpuscles to 0.5 cc. of broth culture, and taking care not to contaminate the broth culture so that it may be used for subsequent tests. Cultures producing little or no hemolysis in two hours at 37 C. may be discarded and subsequent tests omitted. (*b*) A stock culture on a blood agar slant. Capsules should again be looked for after incubation over night. (*c*) Inoculate sodium hippurate broth if it has not already been inoculated directly from the colony. By means of the ferric chloride test hydrolysis of sodium hippurate may be detected in 24 or 48 hours. (*d*) Inoculate glucose broth if it has not already been inoculated directly from the colony. The final hydrogen ion concentration should be determined in not less than 48 hours. (*e*) Inoculate salicin and mannite broths. Saccharose, lactose and raffinose have seldom been found of value for this differentiation. (*f*) Mice may be inoculated, although this test has been found less significant than the others.

To justify condemnation of the milk the streptococcus found should correspond in all respects with the characters of Streptococcus epidemicus as listed in table 1.

STUDIES ON SNUFFLES IN RABBITS

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The resemblance of the rabbit disease known as snuffles to human influenza-like infections has been noted by many. It seems quite likely that light may be thrown on the nature of the human disease by obtaining an understanding of the rabbit infection. The uncertainty that still exists as to the nature of snuffles in rabbits, its bacteriology, its relation to rabbit septicemia, its predisposing factors, and its control make further studies desirable. The confused and scanty literature on the subject has been summarized recently by McCartney and Olitsky [1] and by Webster [2] and need not be reviewed again here.

The principal symptoms of snuffles in the rabbits studied in this laboratory were nasal discharge, coughing and sneezing; diarrhea was observed in some cases. The nasal discharge is commonly serous in the early stage, then gradually changes to mucoid and then to purulent. The forepaws are damp and matted when the discharge is mucoid or purulent, while in the cases of serous discharges this feature may be lacking. In the chronic cases, nasal discharge is persistent, white, tenacious and accompanied with crust formation on the nostrils.

Though the nutrition and general condition seem to be normal in some cases, in others the disease is accompanied by weakness, emaciation, loss of appetite and depilation. The nostrils are often obstructed with the exudate so that dyspnea results. In some acute cases the disease proves fatal in a few days without much nasal discharge or other symptoms. The symptoms may persist for a few weeks to several months, often becoming more severe and ending in death. The mortality from snuffles in the warm season is less than in the cold season, the percentage of infected rabbits in stock less and the symptoms generally lighter. Sometimes the symptoms subside and then develop again. Some rabbits develop a chronic form which endures over a period of several months to a year.

The principal pathologic changes will be described more fully later.

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[1] J. Exper. Med., 1923, 38, p. 591.
[2] Ibid., 1924, 39, p. 837.

Small portions of nasal secretion, blood and lung exudate of snuffles rabbits were put into separate tubes of sheep blood broth and incubated at 37 C. over night. The nasal secretion was taken by swabbing the nasal passage with a sterilized cotton swab which was then dropped into blood broth. A small piece of the lung tissue was cut with sterilized scissors and placed in the medium. After incubation at 37 C. for 12 hours, half of the fluid in each container was filtered through a Berkefeld filter.

Similar dosages of the unfiltered cultures and of the filtrates were injected intraperitoneally into mice, 0.2 to 0.4 cc. per 10 Gm. of body weight. All the mice that died were examined and blood cultures made on plain agar and fresh and heated blood agar. Aerobic and anaerobic cultures were tried. Bact. lepisepticum and B. bronchisepticus were especially sought.

TABLE 1

MICE INJECTED WITH NASAL SECRETION FROM RABBIT WITH SNUFFLES

Symptoms in Rabbits From Which Material Was Obtained	Predominant Bacteria in Nasal Secretion	Result of Injection	
		Nasal Secretion	Sterile Filtrate From Nasal Secretion
Thick discharge, cough	Bact. lepisepticum	Died in 2 days	Lived
Discharge 3 to 4 days, sneezing	Bact. lepisepticum	Died in 3 days	Lived
Purulent discharge, emaciation, sneezing	Bact. lepisepticum	Died in 1 day	Lived
Serous discharge, slight sneezing	Bact. lepisepticum	Ill, but recovered	Lived
Purulent discharge, cough	B. bronchisepticus	Died in 3 days	Lived
Serous discharge	B. bronchisepticus	Slightly ill, but recovered	Lived
Mucoid discharge, sneezing	B. bronchisepticus	Died in 4 days	Lived

Bact. lepisepticum is nonmotile, gives a bipolar stain, and is a slender, gram-negative bacillus. No visible growth is produced on potato medium. Dextrose and saccharose are fermented with no gas; lactose is not fermented (Smith;[3] DeKruif [4]).

B. bronchisepticus is motile, gives bipolar stain and is a slender, gram-negative bacillus. It produces a tan-colored growth on potato medium. It does not ferment dextrose, saccharose or lactose (Ferry;[5] McGowan;[6] Smith [7]).

Table 1 shows that the nasal secretions of snuffles rabbits contain some pathogenic agent for mice, but that this agent did not appear in the filtrates.

[3] J. Comp. Med. and Surg., 1887, 8, p. 24.
[4] J. Exper. Med., 1921, 33, p. 773.
[5] Am. Vet. Rev., 1910, 37, p. 499; J. Am. Vet. Assn., 1912, 68, p. 376.
[6] J. Path. & Bacteriol., 1910, 15, p. 372.
[7] J. M. Res., 1913, 29, p. 291.

Either Bact. lepisepticum or B. bronchisepticus was isolated in great abundance from the nasal secretions, while no organisms were isolated from the filtrates. From the heart blood of the dead mice were isolated the same bacteria which were present in the greatest abundance in the rabbit secretions. No bacteria grew in the anaerobic cultures from the nasal secretions or the filtrates of nasal secretions.

One rabbit (860 Gm.) received intravenously a small dose of nasal secretion broth and died within 24 hours. Bact. lepisepticum was isolated in abundance from the heart blood. Hemorrhage in the lungs was present.

Three typical protocols are given.

1. Buck rabbit, 2,510 Gm., history unknown; thick, purulent discharge, cough, emaciation, matted forepaws. The autopsy showed nasal passages filled with pus; sinusitis, purulent pleurisy and pericarditis, abscess in right lung, hemorrhagic septicemia and pneumonia. Bact. lepisepticum was isolated in large numbers in a pure state from the nasal secretion, blood, lung, exudate and trachea. Cultures of the (unfiltered) nasal secretion in blood broth, lung broth and nasal secretion broth invariably killed mice in from 0.2 to 0.4 cc. doses; filtered material did not kill.

2. Doe rabbit, 1,750 Gm., history unknown, showed emaciation, depilation, slight mucoid discharge, sneezing; died. Serous pleurisy, marked congestion of lungs, hemorrhagic septicemia, peritonitis, pericarditis, B. bronchisepticus isolated in almost pure culture from the nose secretion, lungs and trachea. Broth cultures killed mice precisely as described in protocol 1.

3. Doe rabbit, 2,100 Gm.; mucoid discharge from the nose, sneezing, followed by conjunctivitis, and a purulent discharge; died; paranasal sinusitis, hemorrhagic pleurisy, septicemia, purulent peritonitis, bronchopneumonia. Bact. lepisepticum isolated from the nasal secretion, blood, lungs, sinus, trachea and pleural exudate.

The question whether Bact. lepisepticum or B. bronchisepticus is the real etiological agent of rabbit snuffles was tested by using young cultures of Bact. lepisepticum or B. bronchisepticus incubated at 37 C. for from 18 to 20 hours and suspended in 0.85% salt solution. Mice received intraperitoneally 0.5 cc. of the suspension containing the desired dosage of bacteria. The results were observed five days after injection (table 2).

Necropsies were performed on animals dying after the injection and cultures were made from the heart blood.

One rabbit (1,150 Gm.) which received intravenously 0.02 mg. of Bact. lepisepticum (second cultural generation of blood strain) died 28 hours after the injection. Another rabbit (1,260 Gm.) which received intravenously 0.2 mg. of B. bronchisepticus (second cultural generation of blood strain) died after about 40 hours.

The result shows that both Bact. lepisepticum and B. bronchisepticus isolated from rabbits with snuffles are virulent for mice and rabbits. The virulence of the strains isolated from the blood after death seems stronger than that of the organisms isolated from the nasal secretion during life. The virulence of Bact. lepisepticum was greater than that of B. bronchisepticus.

TABLE 2

MICE INJECTED WITH BACTERIA FROM TISSUES OF RABBITS DYING WITH SNUFFLES

Strain	Bacteria Isolated from Rabbit	Generation of Culture	M L D in Mg. per 10 Gm. of Mouse
Bact. lepisepticum	Nose secretion	3	0.05
	Nose secretion	3	0.1
	Nose secretion	3	0.002
	Blood	2	0.001
	Lung	2	0.001
	Nose secretion	3	0.005
B. bronchisepticus	Nose secretion	3	0.2
	Blood	2	0.01
	Lung	2	0.01
	Nose secretion	3	0.1

TABLE 3

AGGLUTINATION OF ISOLATED STRAINS BY SERUM OF SNUFFLES RABBITS

Strains	Serum of Snuffles Rabbits			
	With Bact. lepisepticum			With B. bronchisepticus
	8	10	11	9
Bact. lepisepticum—tracheal strain	40	80	320	160
lung strain	40	80	80	80
pleural exudate strain	40	40	40	40
Bact. lepisepticum (Washington)	80	40	320	0
B. bronchisepticus (Detroit)	0	160	10	320

The figures give the highest dilutions of serum causing definite agglutination; 0 indicates no agglutination.

Young cultures of these two organisms grown at 37 C. for from 18 to 20 hours were suspended in 0.4% salt solution; 0.1% formalin was added at the rate of about 1.0 mg. per 10 cc. of solution. The suspension used for the test was filtered through paper.

The serum was first diluted with 0.4% salt solution to avoid spontaneous agglutination, but 0.85% solution was later used after ascertaining that we were not dealing with a G. type strain such as De Kruif [8] described. The tubes were incubated at 37 C. and examined after standing over night and again the following day. As a control the serums

[8] J. Exper. Med., 1922, 36, p. 309.

were tested with two typical strains[9] (table 3). None of the cultures agglutinated with normal rabbit serum.

The results show that the serum of snuffles rabbits possesses agglutinin for the isolated strains. The blood from which Bact. lepisepticum was isolated in pure state agglutinates this organism, but not B. bronchisepticus, and vice-versa. Though both strains of Bact. lepisepticum and B. bronchisepticus are closely associated with snuffles in rabbits, the serologic relations differ.

TABLE 4

AGGLUTINATION OF BACT. LEPISEPTICUM AND B. BRONCHISEPTICUS BY IMMUNE SERUM

Strains of Bact. lepisepticum and B. bronchisepticus		Antiserums for			
		Bact. lepisepticum		B. bronchisepticus	
		Washington	Nose Strain 8	Detroit	Strain 9
Bact. lepisepticum	Washington	640	640	0	0
	Nose strain 8	...	640	0	0
	Tracheal strain 8	320	320	0	0
	Blood strain 10	320	640	0	0
	Tracheal strain 11	...	320	0	0
	Nose strain 11	160	320	0	0
	Blood strain 11	320	...	0	0
	Nost strain 1	160	80	0	0
B. bronchisepticus	Detroit	0	20	2560	1280
	Nose strain 9	0	0	640	1280
	Tracheal strain 9	20	10	640	....
	Lung strain 9	..	..	320	....
	Blood strain 9	0	..	640	1280
	Nose strain 5	0	0	320	640
	Nose strain 6	0	0	320	1280

Agglutination tests were made with stock and isolated strains of Bact. lepisepticum and B. bronchisepticus and antiserums for Bact. lepisepticum and B. bronchisepticus.

Rabbits were injected, first subcutaneously, then intravenously, with a salt suspension of the organisms from 18 to 20 hour cultures. A series of three daily injections was followed by a four- to five-day rest and then the injections were resumed, this procedure being continued for several weeks before serum with sufficiently high titer was obtained. Heat-killed suspensions were used for the first injections and the dosage of living cultures was increased up to 10-15 mg. of bacilli. Bleedings from the heart were made the 11th day after the last injections.

The agglutination test was carried out as described (table 4) and the results were practically the same for the stock and recently isolated strains; no definite cross agglutination was obtained.

Since it appears that the pathogenicity of the nasal secretion and other materials from snuffles rabbits is due either to Bact. lepisepticum or B. bronchisepticus, a more detailed examination was undertaken.

[9] Bact. lepisepticum from the Pathological Division of the Bureau of Animal Industry, Department of Agriculture, Washington. B. bronchisepticus from Dr. E. Houghton, Parke, Davis and Company, Detroit.

The nasal bacteria of rabbits have been studied by culturing both nares from the same individuals. If neither Bact. lepisepticum nor B. bronchisepticus was found at the first examination, the examinations were repeated. Since no growth occurred on anaerobic cultivation and none of the filtrates from snuffles materials were pathogenic to mice or rabbits, these two methods of examination were not used further.

Sterilized cotton swabs, one for each side, were inserted into the nostrils of rabbits (1-2 cm. depending on the size of the rabbits). The swabs were rubbed gently over all the nasal mucous surfaces especially the surface of the maxillar turbinates which have many folds and pockets. Fresh and heated blood agar plates (P_H 7.4-7.6)—5% defibrinated sheep blood—were streaked with the swabs. The plates were incubated for 24 to 48 hours at 37 C. Colonies that developed were further studied by smears, hanging drops and cultural tests. Veal infusion agar was later used, since good growth takes place in this medium and the identification of colonies is easier than on blood agar; the growth on the latter, however, is usually more profuse.

A variety of microorganisms was isolated from healthy rabbits; these were in order of their frequency Micrococcus catarrhalis, Staphylococcus albus, gram-negative cocci, Bact. lepisepticum, unidentified organisms, B. bronchisepticus, streptococci.

The two chief pathogenic forms were isolated from the nasal secretion of 110 rabbits with about equal frequency (Bact. lepisepticum, 20%; B. bronchisepticus, 15%). Six of the 22 healthy rabbits from which Bact. lepisepticum was isolated later developed the symptoms of snuffles, and Bact. lepisepticum continued to be demonstrated in their nares. Three of the 17 healthy rabbits from which B. bronchisepticus was isolated developed snuffles at a later period and B. bronchisepticus was isolated from them. All isolated strains of Bact. lepisepticum were DeKruif's type D,[8] type G not being found. Healthy rabbits, showing no clinical signs, later developed snuffles after the injection of toxin or vaccines or after bleeding, etc.; Bact. lepisepticum was isolated from most of these cases. Bact. lepisepticum or B. bronchisepticus isolated from the nasal passages of normal rabbits were pathogenic for mice, but their virulence is low compared to the strains isolated from rabbits with snuffles, the MLD being about 100 times as great as that shown in table 2. Agglutination of these organisms by the serum of the host was usually lacking, never more than 1 : 20.

Examinations of the nasal secretion of snuffles rabbits during life and after death show that Bact. lepisepticum was the predominating organism (table 5).

In some cases Bact. lepisepticum and B. bronchisepticus were demonstrated at the same time in the nasal passage or sinus but never were

both species isolated from the blood, lung or exudate from the same individuals.

A number of virulence and agglutination tests gave the same results as before (tables 2 and 3).

The data presented confirm the previous conclusions that the pathogenicity of the nasal secretion or other materials from the snuffles rabbits may be due either to Bact. lepisepticum or to B. bronchisepticus. There is a close immunologic relation in each case with the respective rabbit serum.

Corresponding to the observation that the virulence of Bact. lepisepticum is greater than that of B. bronchisepticus, the symptoms of snuffles in rabbits from which Bact. lepisepticum is demonstrated are generally more severe than in the B. bronchisepticus types of snuffles, and the mortality of rabbits in the former infection is higher than in the latter.

TABLE 5

BACT. LEPISEPTICUM AND B. BRONCHISEPTICUS PRESENT IN SNUFFLES RABBITS

Number Examined	Condition	Material	Bact. lepisepticum		B. bronchisepticus	
			Number	%	Number	%
60	During life	Nasal secretion	39	65.0	16	26.6
25	After death	Nasal secretion	19	76.0	6	24.0
	After death	Sinus	15	60.0	4	16.0
	After death	Blood	18	70.0	5	20.0
	After death	Lung	19	76.0	6	24.0
5	Killed	Nasal secretion	3	60.0	1	20.0
	Killed	Blood	0	0.0	0	0.0
	Killed	Lung	0	0.0	0	0.0

Nearly all previous investigators of snuffles have worked on acute cases of rabbit septicemia. Attempts were made to produce the acute forms of hemorrhagic septicemia by intravenous, subcutaneous or, in some few cases, intratracheal or intranasal injections with isolated strains. Such acute types are usually fatal, the underlying infection being septicemia or pneumonia. The more subacute, or intermittent, and chronic forms of this disease are commonly present in laboratory stock rabbits.

Concerning the contagious nature of this disease Davis [10] showed that by placing normal young rabbits in intimate contact with typical snuffles rabbits no animals developed snuffles for one or two weeks; even seven months elapsed in some cases before symptoms occurred. All rabbits inoculated subcutaneously with isolated strains died within three to five days of septicemia. From these results he considered that

[10] J. Infect. Dis., 1917, 21, p. 314.

chronically infected animals are not a source of danger to healthy rabbits by infecting them through the respiratory passage, but may be in other ways, as for example through bites. Webster [11] concluded from his experimental induction of the disease that snuffles may be communicable, but that environmental changes influence the result. He found the appearance of snuffles to be associated with the presence of Bact. lepisepticum in the nasal passage.

Intranasal inoculation of these organisms, McCartney and Olitsky [1] found, failed to produce snuffles in rabbits. They did not regard Bact. lepisepticum or B. bronchisepticus as the sole exciting cause. Recently Webster [12] reported that 45 young rabbits were inoculated intranasally with Bact. lepisepticum; 19 (42.2%) died, 16 (35.5%) developed typical chronic snuffles and 10 (22.2%) resisted infection. He considered that it is possible to produce a typical snuffles in rabbits by intranasal injection of Bact. lepisepticum. The results of the inoculation varied according to the natural degree of resistance of the individual animals. Ferry and Hoskins [13] stated that both a chronic form of snuffles and the acute form may be induced by B. bronchisepticus, but remarked that "this statement is not based upon absolute findings—but is the opinion of the authors." There is, therefore, still confusion and difference of opinion regarding the inciting organisms of snuffles.

Since snuffles in rabbits is almost ubiquitous it is not surprising that Bact. lepisepticum and B. bronchisepticus sometimes should be found in normal as well as in infected rabbits. In my experiments most of the rabbits were shown to be free from Bact. lepisepticum and B. bronchisepticus before using.

All animals were carefully observed and weighed and the temperatures taken together with bacteriologic examinations at least two weeks before using.

The body temperature of the animals was lowered by placing them in an icebox or exposing them to cold air. It was thought that by decreasing the animals' resistance snuffles might be induced from a latent sinusitis infection if any existed.

Apparently healthy rabbits which were without symptoms of snuffles but which discharged a small purulent mass on sneezing or when a swab was inserted into the nostrils, were discarded.

It was hoped that chronic snuffles could be produced in normal rabbits by injecting them with strains of Bact. lepisepticum and B. bronchisepticus; therefore, only intranasal inoculations were tried. After several different methods had proved unsuccessful the following one was used: With a long, dull, narrow, silver needle connected to a 2 cc. syringe containing the bacterial suspension, an injec-

[11] J. Exper. Med., 1924, 40, p. 117.
[12] Proc. Soc. Exper. Biol. & Med., 1923, 21, p. 236.
[13] J. Lab. & Clin. Med., 1919, 5, p. 311.

tion was made into the hollow of the opening of the frontal sinus on the inner surface of the nasal bone, care being taken not to injure the mucous membrane of the nose. The suspension was also injected into the pockets among the folds of the maxilloturbinate. It had been demonstrated that methylene blue solution injected by this method entered the frontal sinuses and the pockets among the folds of the maxilloturbinate. The body temperature of the animal was lowered from 4 to 6 degrees F. by placing the rabbit in the icebox for from 1 to 3 hours just before the injection was made.

All animals were examined bacteriologically, and necropsies were made of the animals that died.

TRANSMISSION TESTS

By Contact. Exper. 1.—Three young rabbits were placed in contact with a rabbit having chronic snuffles of two months' duration; the nose secretion of the latter contained Bact. lepisepticum in great numbers. Two of the contact rabbits developed snuffles, one in two weeks and the other in one month, while the third remained well during two months' observation.

Exper. 2.—Two young rabbits were chilled and then placed in contact with a rabbit which had snuffles in an early stage, and from the nares of which B. bronchisepticus had been demonstrated in large quantity. Snuffles resulted in one of these rabbits.

The above experiments, although made with a small number of animals, indicate that snuffles may be transmitted from rabbit to rabbit.

By Inoculation. Exper. 1.—Purulent nose secretions from snuffles rabbits in which Bact. lepisepticum was demonstrated in large numbers.

(*a*) Two rabbits were injected intranasally with a medicine dropper with the secretions mixed in 0.85% salt solution. A slight nasal discharge resulted in both animals on the second day, but this disappeared after 5 and 7 days, respectively.

(b) Two young rabbits were injected intranasally with the same suspension, using the needle method described above. A nasal discharge resulted on the 1st and 2 days and the rabbits died from septicemia on the 23rd and 34th days, respectively.

(*c*) Two rabbits were injected intranasally with the purulent nose secretions. A nasal discharge resulted in both rabbits on the 1st day and one returned to normal after 6 days. The other animal had purulent sinusitis and rhinitis and was killed on the 29th day. Necropsy and bacteriologic studies were performed.

Exper. 2.—(*a*) A culture of Bact. lepisepticum grown for 20 hours on blood agar was suspended in 0.85% salt solution. The MLD, injected intraperitoneally, for mice of 10 Gm. weight was 0.5 mg. Two series of 2 young rabbits received 1 mg. of this culture in the nasal cavity by means of a 2 cc. syringe attached to a medicine dropper. The rabbits in series A were chilled immediately before injection. The inoculated rabbits were then placed in individual cages and observed over a period of 2 months.

In three of the four rabbits a discharge resulted on the first and second days, but all recovered within two to ten days. The symptoms were more severe and of longer duration in the rabbits that were previously chilled than in those which received no treatment previous to injection. The significance of this experimental result remains in doubt.

(*b*) A young culture of Bact. lepisepticum grown on blood agar for 20 hours was suspended in 0.85% salt solution. The MLD, injected peritoneally, for 10 grains mice was 0.1 mg. Two series of young rabbits were inoculated intranasally with 20 mg. of the suspension and observed for 2 months.

All the rabbits developed a discharge, 2 died in 3 to 7 days with pneumonia and septicemia, 3 died after 16 to 32 days with snuffles, 1 had chronic snuffles but lived, and 1 recovered completely after 8 days.

Bact. lepisepticum was demonstrated from the nares, blood, lungs, etc., of the experimentally inoculated animals. The pathologic change in the dead animals was quite similar to that in naturally infected rabbits.

Exper. 3.—Nose secretions from snuffles rabbits containing B. bronchisepticus in large quantity were introduced intranasally with a medicine dropper into a series of rabbits (*A*) and (*B*). Rabbits of the (*B*) series were chilled immediately before inoculation. Three recovered after a discharge of from 2 to 7 days' duration. One developed typical symptoms and finally died on the 39th day with septicemia. The symptoms in the rabbits which were chilled were more severe and of longer duration than in the others.

Exper. 4.—A culture of B. bronchisepticus obtained from the nares of rabbits showing snuffles was grown on blood agar for 20 hours and suspended in 0.85% salt solution. Four rabbits received intranasally 2 mg. of this culture by means of a 2 cc. syringe attached to a medicine dropper. All recovered. Three rabbits received intranasally by the needle method of inoculation 2 mg. of another culture of B. bronchisepticus. Two of the three died. Those inoculated with a needle into the sinuses showed symptoms which persisted for a long period of time. B. bronchisepticus was demonstrated in the rabbits dying with snuffles.

It is thus shown that in selected normal rabbits experimental snuffles was produced which was identical with the snuffles naturally occurring in rabbits. Strains of B. bronchisepticus and Bact. lepisepticum both gave positive results.

LEUKOCYTES IN RABBITS WITH SNUFFLES

The decrease of leukocyte count in the circulating blood of influenza patients in the early stages of the disease is a well recognized fact and is made use of in the diagnosis of influenza. A study was made of the leukocyte count in the blood of rabbits with snuffles to see if there is any correlation between snuffles and influenza in this respect.

The leukocyte count in normal rabbits was first determined before studying it in snuffles. It is well known that in many healthy mammals the leukocyte count varies within wide limits and that there are daily and even hourly changes physiologically under certain conditions. For example, in exercise, pregnancy, sudden exposure to cold, digestion, etc., leukocytosis occurs. Fasting, loss of body heat, shock, etc., are often accompanied by leukopenia.

Twenty-five normal rabbits were examined by the standardized method and the results are as follows:

	Maximum	Minimum	Average
Red blood cells (millions per c.mm.)	5.60	7.77	5.40
Leukocytes (per c.mm.)	5900	12160	8426

These results are similar to those obtained by other recent observers.[14]

The average percentage of the different kinds of leukocytes were as follows:

Lymphocytes	56.4
Mononuclears	4.8
Pseudoeosinophils	36.6
Eosinophils	0.5
Basophils	2.4

These also are very similar to the counts made by others.

The leukocytes in 25 rabbits showing snuffles were similarly examined. The results are shown in table 6:

TABLE 6

LEUKOCYTES IN SNUFFLES RABBITS IN RELATIVELY HEALTHY CONDITION

Type of Infection	Leukocytes, per C.mm.	Percentage of				
		Lymphocytes	Mononuclears	Pseudoeosinophils	Eosinophils	Basophils
Bact. lepisepticum	8,650	50.1	5.2	42.2	0.5	2.0
	6,150	54.5	3.5	41.0	...	1.5
	9,500	46.4	6.7	44.6	0.9	1.4
	12,000	34.7	3.7	59.5	...	3.1
	11,000	52.1	4.5	41.3	0.3	2.1
	14,000	47.6	3.4	46.6	0.4	2.6
B. bronchisepticus	12,900	53.6	6.4	47.3	0.5	2.2
	12,000	34.6	4.5	58.3	0.2	1.9
	9,800	55.0	4.3	37.3	0.3	3.2
	12,500	45.7	3.2	48.3	...	2.8
	9,600	54.6	4.2	38.2	0.5	1.5

TABLE 7

LEUKOCYTES IN SNUFFLES RABBITS IN THE ADVANCED STAGES OF THE DISEASE

Type of Infection	Leukocytes, per C.mm.	Percentage of				
		Lymphocytes	Mononuclears	Pseudoeosinophils	Eosinophils	Basophils
Bact. lepisepticum	16,600	45.9	3.5	48.8	0.5	1.2
	17,800	43.8	4.2	49.2	...	2.8
	9,760*	35.7	3.2	58.5	...	1.5
	4,500*	44.0	1.4	52.1	...	2.5
	9,900*	21.9	2.7	73.8	...	1.4
	13,900*	37.6	3.0	57.3	...	2.1
	8,900*	52.2	3.7	43.0	...	2.1
	18,400	41.5	3.2	53.5	...	1.8
	18,200	46.4	3.8	48.5	...	2.3
	15,800*	48.4	2.1	47.3	...	2.2
B. bronchisepticus	3,400*	38.7	2.0	57.4	...	1.8
	12,500	51.9	2.8	43.8	0.2	1.3
	8,500*	48.8	1.2	47.5	...	1.5
	12,700*	31.4	2.0	65.0	...	1.6
	8,700*	46.8	2.8	47.8	...	2.6
	6,800*	46.2	3.3	47.3	...	3.2

* Rabbit died 2 to 3 days after inoculation.

[14] Fritsch, G.: Arch. f. d. ges. Physiol., 1920, 181, p. 78. Bittner, A.: Folia. Haematol., 1913, 15, p. 237.

The results show that in the blood of snuffles rabbits under relatively healthy conditions there was more or less leukocytosis, involving chiefly the pseudoeosinophils.

The leukocyte count in snuffles in the advanced stages generally increased, especially the pseudoeosinophils, and then usually decreased before death. The number of red blood cells decreased depending on the severity of the symptoms.

Briefly the results are as follows: A slight decrease of the leukocytes in rabbit blood was observed in experimental snuffles in the first few days and then there was an increase. The leukocyte count in the circulatory blood of rabbits decreases in from 10 to 30 minutes after intravenous injection of Bact. lepisepticum, B. bronchisepticus or B. influenzae. It then gradually increases after from 5 to 7 hours and in 24 to 48 hours the leukocyte count has returned to practically normal. The fluctuation was most marked among the pseudoeosinophils.

The leukopenia resulting from the above injections is probably a toxic phenomenon. The blood pressure was decreased with the decreasing of the leukocyte count. Intravenous injections of strains of pneumococci, streptococci, and M. catarrhalis did not produce changes in the leukocyte count in rabbits in the dosage given (0.2 mg.).

SPONTANEOUS SUBCUTANEOUS ABSCESSES

Purulent sinusitis, pleurisy, pericarditis or peritonitis, and sometimes abscesses in lung or liver, etc., are not rare symptoms in snuffles of rabbits. Spontaneous subcutaneous abscesses are often observed.

Schimmelbusch and Mühsam [15] in 1896 described an organism from abscesses in rabbits which on reinoculation produced similar chronic abscesses. Its characters seem to agree with those of Bact. lepisepticum in many respects. Lexer [16] produced osteomyelitis in rabbits by intravenous injection of Bact. lepisepticum in connection with staphylococci. Fuchs [17] isolated a large nonmotile bacillus from the purulent exudate in dead rabbits and produced suppuration by injecting rabbits with this organism. Laven [18] isolated from an abscess in rabbits a small bacillus similar to Bact. lepisepticum, but it did not form acid in dextrose, saccharose or mannite; it was slightly hemolytic and gave a peculiar spermlike odor on blood agar. Davis [19] isolated a small bacillus from

[15] Arch. f. klin. Chirurg., 1896, 52, p. 564.
[16] Ibid., 1896, 52, p. 576.
[17] Cited in Kolle and Wassermann: Handbuch d. path. Mikroorganismen, 1913, 6, p. 181.
[18] Centralbl. f. Bakteriol., I. O., 1910, 54, p. 97.
[19] J. Infect. Dis., 1913, 12, p. 42; 1917, 21, p. 314.

TABLE 8

LEUKOCYTES IN A RABBIT IN THE ADVANCED STAGES OF SNUFFLES

No. of Days	Weight	Temperature	Red Blood Cells (Millions)	Leukocytes per C.mm.	Percentage of Lymphocytes	Mononuclears	Pseudoeosinophils	Eosinophils	Basophils	Remarks
1	990	102.6	5.00	12,000	48.2	4.1	45.7	0.2	1.8	
2	870	103.5	4.40	14,800	47.6	3.8	46.5	0.2	1.9	Very ill, unable to walk
3	820	103.8	4.10	13,200	48.7	3.5	47.8	...	2.0	
7	750	103.2	3.20	9,800	28.5	2.2	60.5	...	2.8	
8	(Died)									

TABLE 9

CHANGES IN LEUKOCYTES IN RABBIT INOCULATED INTRANASALLY WITH B. LEPISEPTICUM

No. of Days	Weight	Temperature	Leukocytes per C.mm	Percentage of Lymphocytes	Mononuclears	Pseudoeosinophils	Eosinophils	Basophils	Remarks
1	1,148	101.2	8,750	58.6	7.2	31.1	0.6	1.5	Before inoculation
2	1,140	102.0	6.100	68.2	5.8	24.5	...	2.5	After inoculation
3	1,060	103.2	10,700	25.0	4.8	65.5	0.2	2.7	
4	986	104.1	18,300	33.0	3.5	60.5	...	3.3	
5	(Died)	102.8	5,800	40.2	3.2	53.5	...	3.1	Ten minutes before death from septicemia

Bact. lepiseptieum was isolated from the blood, lungs and nasal passages.

TABLE 10

CHANGES IN LEUKOCYTES IN RABBIT INOCULATED INTRANASALLY WITH B. BRONCHISEPTICUS

Number of Days	Weight	Leukocytes per C.mm.	Percentage of Lymphocytes	Mononuclears	Pseudoeosinophils	Eosinophils	Basophils	Remarks
1	1,620	8,850	54.3	7.1	36.2	0.6	1.8	Injected 1:15 p. m.
2	.....	6,700	60.0	4.0	33.5	0.5	2.5	Injected 10 a. m.
		7,500	61.8	5.1	29.5	0.6	3.0	Injected 8 p. m.
3	1,550	12,000	44.5	5.1	47.2	0.2	2.5	Slight discharge
5	.....	14,500	43.0	6.2	48.6	0.6	1.8	Increased discharge, slight sneezing
7	.....	13,600	....	...	....	...	...	
9	.....	11,600	50.9	3.7	40.8	0.5	2.1	
12	1,590	9,500	57.3	4.7	34.2	0.3	3.5	Decreased discharge
21	1,640	9,500	57.0	5.2	35.3	0.5	2.0	Slight discharge
22	.....	8,800	....	...	....	...	...	Aggl. titer of serum against B. bronchisepticus, 1:320
24	1,650	8,600	56.3	5.6	35.5	0.5	2.1	Exposed in cold air (outdoors) 3 hours
25	.....	19,800	38.6	4.8	53.7	...	2.8	Increased discharge, sneezing, cough
26	.....	17,500	42.4	4.5	49.9	...	3.2	
27	1,500	18,160	41.8	4.2	50.5	...	3.5	Purulent discharge, very ill
28	.....	21,000	38.8	3.0	54.6	...	3.6	Very ill
30	1,380	11,500	40.2	4.1	51.6	...	4.1	Very ill
31	(Died)							

B. bronchisepticus was demonstrated from the nose secretion, blood and lungs.

an abscess in rabbits and thought it apparently identical with the bacillus of rabbit septicemia. He produced similar abscesses in rabbits by subcutaneous injection with this strain and with a rabbit septicemia strain, and also produced septicemia in rabbits by giving an intravenous injection with the abscess strain. The organism seems to be identical with Bact. lepisepticum but it did not produce indol and agglutination was not caused by the serum of infected animals.

Spontaneous subcutaneous abscesses were observed in four rabbits, two afflicted with snuffles, the other two free.

Adult Rabbit 4.—Aug. 1: A subcutaneous abscess the size of a grape was noticed on the neck. Aug. 15: Increased in size; one more abscess formed. A slight watery discharge from the nostril and sneezing. Aug. 25: The abscess increased to the size of a walnut and the general symptoms of snuffles more prominent. Bact. lepisepticum was demonstrated from the abscess and the nasal secretion. Sept. 5: One of the abscesses broke and discharged. The serum agglutinated the isolated strain at 1:40. Sept. 15: Died. Subcutaneous abscess on neck; nasal passage and bronchi filled with pus, purulent pleurisy, pericarditis, bronchopneumonia, hemorrhagic septicemia. Bact. lepisepticum demonstrated in pure culture from the blood and purulent exudates.

Adult Rabbit 80. Nov. 25: An abscess about the size of a sparrow egg noticed in the left breast; no symptoms of snuffles; Bact. lepisepticum demonstrated in a pure state from the abscess, but not from the nares. Jan. 3: The serum agglutinated the isolated strain at 1:80. Jan. 7: Killed; no noticeable changes. Bact. lepisepticum demonstrated in a pure state from the abscess, but not from the lungs or blood.

Young Rabbit 57.—Jan. 28: A subcutaneous abscess about the size of a grape was noticed on the left side; Bact. lepisepticum demonstrated in pure state; no sign of snuffles. Feb. 10: Abscess enlarged to size of a walnut. Feb. 25: Incision and drainage. March 1: Recovered. The serum agglutinated the isolated strain at 1:80.

Adult Rabbit 47.—March 20: A small abscess noticed on the back and a slight discharge from the nostril. Bact. lepisepticum demonstrated from the abscess and the nasal secretion. April 10: The abscess now size of walnut; distinct symptoms of snuffles. May 10: Died; subcutaneous abscess on back; bronchopneumonia, hemorrhagic septicemia, purulent pleurisy and peritonitis. Bact. lepisepticum demonstrated in pure culture from the abscess, blood, lung and purulent exudates. The serum 2 days before death agglutinated the isolated strain at 1:40.

In these few cases all the abscesses occurred in the loose subcutaneous tissue and all were distinctly encapsulated. The subjacent structure was not invaded by it except in rabbit 47. The wall of the abscesses was usually a few mm. thick and consisted of connective tissue easily broken; it was at first free from the skin; later adherent, depending on the size. The content was at first highly purulent and quite moist so that it might be taken out easily with a syringe, but later it became yellowish gray and cheesy in appearance.

In a stained smear many phagocytes were observed with numerous small bacilli in all stages of disintegration. Bact. lepisepticum was demonstrated in pure culture in all these abscesses.

The strains isolated from the abscesses were identical with Bact. lepisepticum.

The strains isolated from the blood, liver, lung and pleural exudate behaved in a similar manner. The virulence of the abscess strains is not as high as the strains isolated from the blood or lungs.

TABLE 11

AGGLUTINATION OF ABSCESS STRAINS BY B. LEPISEPTICUM ANTISERUM

Abscess Strains	Highest Dilutions of Serums	
	(W)	(9)
45	320	640
30	160	320
57	320	640
47	320	640
Bact. lepisepticum W	320	640
Bact. lepisepticum 9	320	640

TABLE 12

VIRULENCE OF BACT. LEPISEPTICUM STRAIN FROM ABSCESS

Strains from	Culture Generation	M L D in Mg. per 10 Gm. of Mouse
Abscess of rabbit 4	5	0.05
Lung of rabbit 4	5	0.01
Abscess of rabbit 47	3	1.0
Lung of rabbit 47	3	0.05
Blood of rabbit 47	3	0.05

Three rabbits were injected with strains from abscesses or from the lungs of snuffles rabbits.

May 12: Adult rabbit injected subcutaneously in the flank with 9.5 mg. of lung strain from snuffles rabbit (47). May 15: A small consolidation the size of the finger tip was noted in the infected portion. May 18: Inflammation accompanied by intense edema in that portion. May 22: Inflammatory process spread rapidly toward abdominal wall. May 27: Died; subcutaneous phlegmon in the side; serous pleurisy; bronchopneumonia, hemorrhagic septicemia, serous peritonitis. Bact. lepisepticum demonstrated in pure state from the inflammatory portion, blood and exudate.

No abscesses were produced in two animals, the animals dying of septicemia after 15 days.

Two rabbits were injected subcutaneously with 0.3 mg., respectively, of a lung and abscess strain of rabbit (47). Both produced similar abscesses to those occurring naturally, and attained the size of a walnut after 45 days. Bact. leptisepticum was demonstrated in a pure state in the contents. After incision and drain-

age both rabbits recovered. Snuffles was produced in one young rabbit (49) by injecting it intranasally with an abscess strain from rabbit (47).

The cases are too few in number to warrant conclusions, but it would seem that the abscess strains are apparently identical with Bact. lepisepticum isolated from snuffles rabbits, and that both abscess and lung strains possess the ability to produce acute purulent inflammation with abscess formation.

RELATION BETWEEN SNUFFLES IN RABBITS AND THE ASSOCIATED SINUSITIS

Paranasal sinusitis is a common symptom in snuffles in rabbits as previous investigators have noted. Bact. lepisepticum and B. bronchisepticus have been frequently found in the affected sinuses. Ferry and Hoskins [20] demonstrated Bact. lepisepticum in 33.3% and B. bronchisepticus in 42.8% of the sinuses of 21 killed rabbits showing snuffles. Recently McCartney and Olitsky [1] reported that practically all stock rabbits showing snuffles and 9% of apparently normal rabbits exhibit chronic inflammatory processes in the upper nasal passages associated with chronic sinusitis.

The opinion that neither Bact. lepisepticum nor B. bronchisepticus can be considered the essential incitant of experimental snuffles in ordinary stock rabbits is held by many investigators because of the preexisting nasal sinusitis in the animals. These investigators, however, have failed to note any other causative agent of sinusitis.

The frequent occurrence of sinusitis associated with an affection of the upper nasal passages suggests that there may be some anatomic reason which may explain this sinusitis.

The anatomic construction of the nose of the rabbit is much more complicated than that of man; for example, the turbinate bones of man are relatively smooth and normally without noticeable convolutions, while all of the turbinate bones of the rabbit are greatly convoluted, especially the maxilloturbinate. This occupies the anterior portion of the lateral wall of the nose and consists of numerous fine rigid elevations, producing many folds and pockets covered with the nasal mucous membrane. The ethmoturbinate, which has much broader folds, is placed posteriorly.

The opening into the frontal sinus can be easily reached by a straight passage along the inner surface of the nasal bone. This sinus

[20] J. Lab. & Clin. Med., 1919-1920, 5, p. 311.

can, therefore, be infected with a solution much more easily than in man. The ethmoid sinus, the maxillary sinus and the nasolacrimal ducts also open into the nasal passages as in man.

Due to the peculiar anatomic construction of the rabbit's nose, the swelling of the mucous membrane by congestion or nasal catarrh may convert the folds or sinuses into closed cavities and thus permit an infectious process or purulent material to persist locally for a long time before symptoms develop. This may be the cause for the chronic nasal affection in rabbits. Fox [21] makes the following statement: "A dissection of the accessory nasal sinuses exposes the relatively large size of these spaces in the lower mammals, and the capacious opening into the nasal cavities. . . . The extent of the turbinate and the richness in mucosa is probably greater in all mammals than in birds. . . . It would seem that rhinitis occurs more often in birds with their small sinuses and channels while sinusitis and general infection occur more often in mammals with their large sinuses and extensive turbinate apparatus."

Ballenger [22] states that about one half of the cases of maxillosinusitis in man are infected from the teeth rather than from the nose, while the other sinuses are nearly always infected from the nose.

Owing to the fact that in the rabbit the frontal sinus is easily entered from the nose, this sinus in rabbits is more subject to infection than is the maxillary sinus, especially during the early stages of snuffles. At this time either Bact. lepisepticum or B. bronchisepticus or both organisms could be demonstrated as more abundant on the surface or pockets of the maxilloturbinate and frontal sinus than in other places.

Sinusitis often follows influenza in man, producing a pathologic change similar to that caused by pneumonia. Gerber [23] observed that paranasal sinusitis in man frequently followed influenza. In the etiologic study of 210 cases of frontal sinusitis in man, he found coryza to be the cause in 91, influenza in 61, and other acute or chronic infectious diseases in 58 cases. In the frontal or maximal sinus he often observed suppurative processes during influenza and it was not rare that such affections were discovered at necropsy, while they were overlooked during life.

Fraenkel [24] found, after a careful examination of the paranasal sinuses of 60 influenza patients, that only 16 patients were normal, the

[21] Disease in Captive Wild Mammals and Birds, 1923, pp. 135, 136.
[22] Diseases of the Nose, Throat and Ear, 1911, p. 166.
[23] Med. Klinik., 1919, 15, p. 427.
[24] Deutsch. med. Wchnschr., 1910, 45, p. 89.

remainder, i. e., 73.3%, showing more or less affection. In 22 cases, B. influenzae was found, but in a pure state in only five of the cases.

Prym [25] reported that 71 of 92 sphenoid sinuses of influenza patients which were examined varied from slight inflammation of the mucous membrane to complete purulent processes.

A bacteriologic examination was made of the sinuses of 25 rabbits showing snuffles. The results may be summarized briefly as follows: Of 25 rabbits dying from snuffles, Bact. lepisepticum was found in 19 (76%), and B. bronchisepticus was found in 6 (24%) in a pure state in great abundance in the nose, blood, lungs, etc. In 21 animals (1) a purulent exudate was present in 16 frontal sinuses and in 14 maxillary sinuses; (2) Bact. lepisepticum was found in 15 (71%) of the frontal sinuses and in 13 (61%) of the maxillar sinuses; (3) B. bronchisepticus was found in 4 (19%) of the frontal sinuses and in 4 (19%) of the maxillar sinuses.

Of 7 rabbits dying from other causes chronic sinusitis was observed in one case; Bact. lepisepticum was demonstrated from it. B. bronchisepticus was found in another animal in which there was no sinusitis.

Eleven rabbits were injected, 9 with Bact. lepisepticum, 2 with B. bronchisepticus. All but one (Bact. lepisepticum) showed purulent sinusitis and purulent exudate was found in 9 frontal sinuses, and in 7 maximal sinuses. In the one animal not developing sinusitis, pus containing Bact. lepisepticum was found in the lung.

Both natural and experimental snuffles begin with sneezing and a slight watery discharge from the nostrils, with a gradually increasing discharge of mucoid or purulent material; this condition may be followed by general symptoms and finally develop into a fatal septicemia. In some cases the disease is prolonged with a purulent discharge and slight sneezing, the animals returning to a healthy condition after a number of days, or the infection changing to the intermittent or chronic form. In the acute cases, death may result in a few days with septicemia and without noticeable nasal discharge or other local symptoms.

It is often observed that in healthy rabbits showing only slight symptoms of discharge or sneezing severe symptoms may be brought on suddenly by lowering their resistance. The injection of dead bacteria or of other foreign protein, or exposure to very cold or hot air, or bleeding may lower resistance. Sometimes apparently normal rabbits without any sign of snuffles develop snuffles when their resistance is

[25] Ibid., 1919, 45, p. 880.

lowered. As McCartney and Olitsky [1] reported, when the animal's resistance is lowered, the long-standing inflammatory process can flare up into an acute exacerbation, and then show itself as typical snuffles.

In cases of long duration, all the sinuses were found filled with thick, white, tenacious, odorless pus, but in cases of very short duration only a swelling of the mucous membranes was noticed.

Bact. lepisepticum or B. bronchisepticus were often found on the surface of the maxilloturbinate, especially in the pockets among its folds which were always covered or filled with mucoid secretion or purulent exudate. In nearly all cases, the mucous lining of the maxilloturbinate was swollen and reddened.

In stained films of the purulent exudate, polymorphonuclear and mononuclear cells were observed with small bacteria present, mostly in various stages of disintegration.

As sinusitis in man was observed to be high during the influenza pandemic, so sinusitis in rabbits associated with infection of the nasal passages was observed in most cases of snuffles (68%) in the laboratory stock rabbits and in 14% of healthy rabbits.

Snuffles in rabbits begins generally with nasal discharge and then produces sinusitis and pulmonary symptoms. On the other hand, sinusitis is sometimes observed in healthy rabbits without any signs of snuffles. Therefore, sinusitis in rabbits is a beginning symptom of snuffles, but it is often also present as a latent affection.

Bact. lepisepticum and B. bronchisepticus are the organisms found to be most predominant in rabbit sinusitis. The isolated strains are apparently identical with the snuffles strains of the same organisms.

SUMMARY

The nasal secretions, blood and lung exudates from rabbits with snuffles contain some organism pathogenic for mice and rabbits, but the filtrates from these materials are not pathogenic. Bact. lepisepticum was isolated in pure culture in the majority of cases; B. bronchisepticus was also isolated in pure culture, but in fewer cases.

Other bacteria were observed in less abundance in the blood or exudates of snuffles rabbits. From the heart blood of animals dying after being injected with snuffles material, either Bact. lepisepticum or B. bronchisepticus was always isolated.

For mice and rabbits the virulence of Bact. lepisepticum and B. bronchisepticus is high, the latter, however, being less virulent than the former.

The strains of Bact. lepisepticum or B. bronchisepticus from snuffles rabbits show a close relationship immunologically to the serum of those rabbits in which identical organisms were demonstrated in abundance, while other organisms less numerously present do not show such a relationship.

The isolated strains of Bact. lepisepticum and B. bronchisepticus are agglutinated by the respective antiserums, but do not show cross agglutination. The biologic characters of the isolated strains of Bact. lepisepticum or B. bronchisepticus are identical with those of the type strains received from other laboratories.

Snuffles identical with natural snuffles was produced in rabbits experimentally with strains of either Bact. lepisepticum or B. bronchisepticus.

The leukocyte count in the rabbit's blood was found to decrease immediately after injection with Bact. lepisepticum, B. bronchisepticus or B. influenzae, remaining low for a few hours and then increasing so as to reach normal within 48 hours. This fluctuation was conspicuous in the pseudoeosinophils.

In four spontaneous subcutaneous abscesses, Bact. lepisepticum was demonstrated in pure culture; the abscess strains were identical with Bact. lepisepticum strains from snuffles rabbits. The serum of rabbits having abscesses possessed a certain amount of immune agglutinin for the autogenous strain and for stock strains of Bact. lepisepticum. Subcutaneous abscesses were produced experimentally by the injection of an abscess strain and of a lung strain of Bact. lepisepticum. B. bronchisepticus was not found in the abscesses in these cases.

Sinusitis accompanied most cases of rabbit snuffles. Bact. lepisepticum and B. bronchisepticus were the predominant organisms in the sinusitis, the strains appearing to be identical with the snuffles strains of these organisms.

ANATOMIC STUDY OF RABBIT SNUFFLES WITH SPECIAL REFERENCE TO ETIOLOGY, AND CORRELATION WITH EXPERIMENTAL PFEIFFER BACILLUS PNEUMONIA AND HUMAN INFLUENZAL PNEUMONIA

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I have shown [1a] that Bact. lepisepticum and B. bronchisepticus have an etiologic significance in snuffles and that symptoms identical with the naturally acquired disease could be induced in healthy rabbits by intranasal inoculation with these organisms.

In this paper, the pathologic changes produced in both natural and experimental snuffles are described and these changes correlated with the bacteriologic findings. The lesions observed in rabbits are compared with those of human influenzal pneumonia and with experimental pneumonia in rabbits induced by B. influenzae. The pathologic material was obtained from rabbits with naturally infected sub-acute or chronic snuffles as it is ordinarily observed in laboratory stock and from those with the experimentally induced disease of over two weeks duration. Saelhof [1b] has recently investigated this disease but apparently has not distinguished between Bact. lepisepticum and B. bronchisepticus, preferring to use the general group name, B. bipolaris.

PATHOLOGY OF SNUFFLES CAUSED BY BACT. LEPISEPTICUM

Natural Infections.—Necropsies were made on twenty cases of subacute or chronic types. Histologic studies were made on the organs of fifteen of these. All the animals showed the typical symptoms of snuffles: nasal discharge, frequent sneezing, cough, mottled fore paws, loss of appetite, etc. All died from a septicemia. Bact. lepisepticum was present in pure culture in the nasal secretion, sinus, blood, lung, trachea and exudate. The serum agglutinated the isolated strains in moderate dilutions. The necropsies were performed from ½ to 15 hours after death. Sections for microscopic study were taken from the lungs,

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[1a] J. Infect. Dis., 1926, 38, p. 389.
[1b] J. Infect. Dis., 1921, 28, p. 374.

trachea, heart, liver, spleen, and kidneys, fixed in Zenker's fluid, sectioned by celloidin, and stained with hematoxylin and eosin. Bacteria in tissue were stained by anilin water methyl violet. The findings are summarized below.

Macroscopically, the nasal passages were covered with mucoid or purulent secretion, the nasal passages often being obstructed with pus. The nasal mucosa was swollen and intensely red, especially on the turbinates. In the pockets among the folds of maxilla turbinate, thickly purulent secretion was often found, also within the sinus—even in the mild cases, when purulent secretion was not noticed on other portions. The blood vessels were generally congested.

Sinuses: Purulent sinusitis was often observed and in the severe cases all the sinuses were filled with pus. The mucous membrane was red and swollen. Hemorrhages were not found in the sinuses.

Trachea: The trachea often contained a slimy mucoid or frothy, slightly blood-tinged fluid, and generally the inflammation had extended from the nasopharynx down into the trachea and bronchi.

Lungs: The lungs were slightly increased in size and did not collapse completely as usual, and sometimes not at all, depending upon the size of the affected area. The surface showed dark bluish or reddish consolidated areas of a mottled appearance, usually scattered, small and sharply defined from the surrounding pink tissue.

Often a large part of a lobe showed confluent bronchopneumonia; sometimes quite marked in the posterior portions of the lower lobes. But the affection was not limited to one lobe, often including whole lobes of both lungs. The affected areas were found to be the patches of increased resistance observed on palpation, but not so prominent, even in confluent form, as in lobar pneumonia. Compensatory emphysema was often observed in the lungs between the consolidated portions. Crepitation was felt on careful palpation of the surrounding lung tissue between the consolidated patches.

The pleural surface was often covered with a thin layer of cellular deposit, occasionally a purulent mass (in six of ten cases) and often was slightly adherent to the thoracic surface of heart sac or diaphragm, according to the extent of the inflammatory process. In some cases the pleura was remarkably thickened. Serous exudate was usually found in the pleural cavities; occasionally a purulent exudate, or bloody fluid. Very little fibrin was observed, even in severe cases.

A cut section of the lung appeared moist and edematous with frothy, dark red fluid. The consolidated areas projected from the surface, vary-

ing in size from that of a pin head to that of a pea, and were generally dark red. Bloody fluid could be squeezed from the patches of consolidation. In the cases of short duration, small patches near the surface surrounded by a zone of hyperemia were commonly found, but in the more chronic form consolidation became confluent in character covering a large area, and situated around a central bronchiole. Often necrotic areas, and sometimes abscesses, were observed in the center of the consolidation. In one case an abscess filled about half of one lower lobe. Remarkable congestion of all pulmonary vessels was constantly observed. Bronchi and bronchioles usually contained a slimy, frothy, reddish fluid and occasionally were filled with pus. The tracheal and peribronchial lymph glands were often considerably enlarged.

Heart: The surface of the heart was often covered with a fibrinous exudate. In five out of ten cases this was observed from a slight to an intense degree. Associated with it, a cloudy, slightly fibrinous exudate, even purulent in one case, was found in the pericardial cavity. No noticeable change of valves or endocardium was found.

Abdominal cavity: In one case purulent exudate was observed in the peritoneal cavity. All the surfaces of the liver, peritoneum and intestines were covered with a purulent fibrinous exudate and intestines were closely adherent. The large intestines in two cases were found to have a watery content, and the mucous membrane appeared edematous. The spleen was occasionally slightly enlarged.

The results obtained appear to be almost the same as those reported by Saelhof [1b] except that he did not find pericarditis. He did not mention purulent pleuritis, which so often occurred in my cases. This may be due to the fact that in most cases his animals succumbed after a short duration of the disease (from 5 to 7 days usually, and in some cases within 6 to 48 hours) whereas the rabbits employed in this experiment lived a much longer time.

The microscopic findings are as follows: Lungs: All sections of the lungs showed bronchopneumonia quite constantly, accompanied usually by lymphangitis and interstitial inflammation. Simple hemorrhage, hemorrhagic edema, and hemorrhagic bronchopneumonia were observed frequently in cases of varying severity. Edema was a constant feature; purulent pleuritis with pronounced subpleural hemorrhage accompanied the acute cases.

Perivascular and peribronchial lymphatic infiltration occurred constantly, leukocytes being often present. The intima and adventitia of the blood vessels were edematous very frequently, and always in the

advanced stages. A striking feature was the not infrequent sub-intimal leukocytic infiltration of the large arteries having perivascular lymphangitis. Thrombi were not infrequently observed.

Suppurative bronchitis and hyperplasia of bronchial lymph nodes were frequently observed and occasionally hyperplasia of tracheal epithelium. Abscess formation was found in the advanced stages. The bronchioli were filled with polymorphonuclear leukocytes and lymphoid cells and often red blood cells were mixed with them. The epitheliar cells of the bronchioli were often partly or completely desquamated and underlying tissues were markedly infiltrated with leukocytes. The capillaries of the adjacent interstitial tissue or contiguous alveoli usually showed engorgement. As before mentioned, the engorgement of the peribronchial lymph channels with leukocytes was often a conspicuous feature.

The alveoli contiguous to the bronchioli contained red cells, desquamated alveolar epithelium or leukocytes with coagulated plasma extravasal, but not a mass of fibrin as in croupous pneumonia. The alveolar epithelial cells were swollen and edematous and their outlines were indistinct, often imperceptible, especially in the cases becoming confluent with subsequent obliteration of the individual alveoli. Alveoli further distant from the bronchioli contained coagulated plasma, but were mostly free from exudate. In the centers of consolidation necrosis often occurred and sometimes abscess formation was observed. The affected portions were usually found in spots, but sometimes, especially in advanced cases, included large areas by confluence. The alveolar capillaries were greatly engorged in nearly every case. Vesicular emphysema was often present, and was especially marked between the affected portions.

Many phagocytes, phagocytized red cells, cellular debris, small bacilli, and polymorphonuclear leukocytes, were found in the stained films made from the blood and exudate of the affected portions of the lung. In tissues stained for bacteria many small bacilli, free or contained in phagocytes, were found in the bronchi, bronchioles, alveoli, interstitial cells, purulent exudates, and hemorrhagic portions.

Heart: The hearts showed pericarditis, sometimes purulent—one case presenting the picture of an acute diffuse suppurative myocarditis.

Liver: Little pathological change was observed in the livers except for some with coccidiosis. A few contained blood pigment, a few showed hemorrhage, and one had an abscess. An increase in fat content was observed in some cases.

Spleen: The spleens occasionally showed endothelial hyperplasia and abscess formation.

Kidneys: The kidneys infrequently showed slight change in tubular epithelium or slight hemorrhage.

Experimental Infections.—As described in the previous paper, snuffles was experimentally produced in ten normal rabbits by intranasal injections of cultures of Bact. lepisepticum and three with the nasal secretion of a snuffles rabbit from which Bact. lepisepticum was isolated; eight of these were examined after death and sections were made from various organs. Bact. lepisepticum was isolated from the nasal secretion, blood, lung, or exudate of all these animals.

TABLE 1

SUMMARY OF PATHOLOGIC CHANGES WITH EPERIMENTAL BACT. LEPISEPTICUM INFECTIONS

Days of Sickness	Principal Pathologic changes
21	Nasal passage, sinus, bronchi filled with pus; bronchopneumonia, hemorrhagic edema, ensuing emphysema
33	Nasal passages, sinus, bronchi filled with purulent material; bronchopneumonia, edema, hemorrhagic thickened alveolar walls; emphysema
28	Killed on 29th day. Right frontal and maxillary sinus filled with pus (injected side). No noticeable change in lung
25	Bronchopneumonia, lymphangitis, hemorrhage, edema; sinusitis
28	Sinus, nasal passage filled with pus; bronchopneumonia, hemorrhage, edema
3	Slight frontal sinusitis, serofibrinous pleurisy, pericarditis; bronchopneumonia, hemorrhage, edema; bronchi filled with pus
7	Sinusitis (injected side); purulent pleurisy, fibrinous pericarditis; bronchopneumonia, hemorrhage, edema
35	Sinusitis, purulent fibrinous pericarditis; bronchopneumonia, hemorrhage, edema

The pathologic changes in the experimental snuffles were similar in all respects to those of the naturally infected rabbits. It is therefore possible to reproduce snuffles by intranasal injections with nasal secretions containing Bact. lepisepticum or with pure cultures of this organism.

PATHOLOGY OF SNUFFLES CAUSED BY B. BRONCHISEPTICUS

Necropsies were made on 8 rabbits; 5 of these were naturally infected and 3 experimentally. Sections were prepared from the various organs of these animals. B. bronchisepticus was isolated from the nasal secretions, blood, lungs, trachea, and exudate from all the rabbits. The principal pathologic changes are shown in the following summaries:

The pathologic changes in experimental B. bronchisepticus snuffles are apparently similar in all respects to the naturally acquired disease which appears to be caused by B. bronchisepticus.

The macroscopic pathologic changes of B. bronchisepticus snuffles resembled in many respects, especially in hemorrhage, edema, perivascular or peribronchial edema in the lungs, those of Bact. lepisepticum snuffles, but scattered consolidated patches on the surface of the lungs were rarely found. In one out of five naturally infected rabbits the changes of slight bronchopneumonia were observed, but not in the three of experimental bronchisepticus snuffles. Hemorrhage, engorgement, and edema in the lungs were more marked than in the lepisepticum pneumonia. Serous or serofibrinous pleurisy was observed in a few

TABLE 2
PATHOLOGIC FINDINGS WITH B. BRONCHISEPTICUS INFECTIONS

	Duration of Sickness	Principal Features
Natural Infections	All more than 10 days; onset unknown	Sinusitis. Lungs: Hemorrhage, edema, ensuing emphysema; perivascular and peribronchial round cell infiltration; hyperplasia lymph follicles around large bronchi; alveolar walls thickened, bronchitis
		Lungs: Hemorrhage, edema, atelectasis, emphysema; perivascular and peribronchial leukocytes and infiltration; thickened alveolar walls, bronchitis, serofibrinous pleuritis and pericarditis
		Sinusitis. Lungs: Hemorrhage, atelectasis, slight emphysema, bronchitis, perivascular edema; thrombi in arteries; alveolar walls thickened
		Bronchopneumonia. Hemorrhage, edema; bronchioles and blood vessels filled with blood, thrombi in small blood vessels; perivascular infiltration; bronchitis
		Sinusitis. Lungs: Bronchitis, emphysema, atelectasis, edema, hemorrhage, lymphangitis
Experimental Infections	38	Lungs: Edema, purulent exudate in large bronchi and desquamation of bronchial epithelium, perivascular round cell infiltration, edema of adventitia of large blood vessels, hemorrhage
	19	Lungs: Hemorrhage, edema, emphysema, perivascular edema, edema of adventitia of large blood vessels
	31	Sinusitis. Lungs: Hemorrhage, edema, emphysema, congestion, alveolar walls thickened, large bronchi filled with pus, perivascular edema, desquamation of bronchial epithelium

cases, but purulent exudation was not found in the pleural, pericardial or peritoneal cavities.

On section, purulent fluid was sometimes found in the lumen of the bronchi or bronchioles, but no plugging with purulent masses in the bronchi or bronchioles, as was often observed in the lepisepticum pneumonia. No abscesses were found.

Other changes in the internal organs were similar in all respects to those in the lepisepticum cases.

Microscopical changes were also very similar to those in lepisepticum snuffles. Hemorrhagic edema and hemorrhage occurred constantly. The walls of the alveoli were frequently thickened, the capillaries distended with blood. Emphysema was very often present, and occasionally

atelectasis. The picture of bronchopneumonia was rarely observed. Desquamation of the bronchial epithelium was a common feature, and the large bronchi were often filled with leukocytes. Occasionally edema of the adventitia and intima of the blood vessels was observed. In a few instances a subendothelial infiltration of leukocytes and lymphocytes was present in the blood vessels. In the alveoli, red blood cells were often found with coagulated plasma or serous exudate, but cellular infiltration was rare.

COMPARATIVE STUDY OF BOTH TYPES OF SNUFFLES

As above described, the pathologic changes of snuffles in rabbits as caused by Bact. lepisepticum and B. bronchisepticus were similar in many respects, but there are several marked differences. For example, there was more frequent occurrence of abscesses, bronchopneumonia, purulent pleuritis, and marked lymphangitis in the former, while in the latter, hemorrhage and hemorrhagic edema were more common. In general, cellular infiltration and the tendency toward suppurative inflammation were more marked in the former while in the latter serous exudation appeared more frequently. Thickening of the alveolar walls was more commonly observed in the latter.

Bact. lepisepticum resembles B. influenzae in many respects, although the former is not hemophilic. Levinthal [2] described the three characteristic effects of B. influenzae as "toxic, phlogistic and hemorrhagic." This statement would apply equally well to Bact. lepisepticum. Furthermore, in symptomatology and epidemiology a striking similarity exists between rabbit snuffles caused by Bact. lepisepticum and influenzal pneumonia. In this type of snuffles the principal pathologic features were bronchopneumonia, hemorrhage, intense engorgement, edema, ensuing emphysema, bronchitis—often purulent—frequent purulent pleurisy and sometimes central necrosis or abscess in the consolidated portions of the lungs.

THE CORRELATION BETWEEN SNUFFLES PNEUMONIA IN RABBITS CAUSED BY BACT. LEPISEPTICUM, HUMAN INFLUENZAL PNEUMONIA AND EXPERIMENTAL B. INFLUENZA PNEUMONIA IN ANIMALS

Microscopically, in Bact. lepisepticum snuffles pneumonia in rabbits, there was dilatation of capillaries with red blood cells, serous and hemorrhagic exudate, dilatation of bronchioles and alveoli, partial or some-

[2] Levinthal, Kuczynski, Wolff: Pandemie von die Grippe, 1918, 1921.

times complete desquamation of epithelium in bronchi, pus in bronchi and bronchioles, infiltration of leukocytes in neighboring alveoli and alveolar walls, serous exudate and round cell infiltration around blood vessels and bronchioles, thickened pleural walls with infiltrated cells. Often there was also a fibrinous deposit on the pericardium.

According to Pfeiffer,[3] MacCallum,[4] and Wolbach,[5] who studied influenzal pneumonia in man, the following peculiar lesions are characteristic: intense engorgement, hemorrhage and edema, purulent bronchitis, scattered foci of peribronchial consolidation and ensuing emphysema and bronchiectasis, and bronchopneumonia. Microscopically, there is serous and hemorrhagic exudate, dilatation of bronchioles and alveoli, partial destruction of bronchial mucosa, pus in bronchi and bronchioles, round cell infiltration and thickening of bronchial walls, infiltration of leukocytes in neighboring alveolar walls, leukocytes and desquamated epithelium in adjacent alveoli and deposit of hyaline material on alveolar walls. Le Count[6] also called attention to the occurrence of focal capillary necrosis in the alveolar walls.

Cecil and Blake[7] experimentally induced in monkeys a pathological change similar in all respects to the spontaneous B. influenzae pneumonia in man, by intranasal or intratracheal inoculation with B. influenzae. Recently Oguchi[8] has reported a similar experimental B. influenzae pneumonia in rabbits. Rhinitis and sinusitis are common symptoms of snuffles in rabbits, and Cecil and Blake[7] observed these also in five of nine monkeys inoculated intranasally with B. influenzae.

The pathological changes in snuffles pneumonia caused by B. lepisepticum, therefore, resemble in many respects those of B. influenzae pneumonia in man and experimental B. influenzae pneumonia in animals. But the pneumonia of the former differs in one respect from that of the latter. Perivascular infiltration is commonly observed in the former and the tendency toward suppurative inflammation and bacteremia appears to be more frequent in the former than in the latter. Oguchi[8] observed, in his experimental B. influenzae pneumonia, marked perivascular inflammation, often complicated by pleurisy with serous exudate. Many animals died from intratracheal infection. Bacteria were found in the blood after death. Cecil and Blake also reported similar results. This corresponds closely to snuffles pneumonia.

[3] Ztschr. f. Hyg. u. Infectionskrankh., 1893, 13, p. 357.
[4] Monograph, Rockefeller Inst. for Med. Res., 1919, 31, p. 428.
[5] Bull. Johns Hopkins Hosp., 1919, 30, p. 104.
[6] J. Am. Med. Assn., 1919, 72, p. 1519.
[7] J. Exper. Med., 1920, 32, pp. 691, 719.
[8] T. Keio Egaku, 1923, 3, No. 1.

The characteristic changes in the human influenzal pneumonia—hemorrhage, serous exudation, appearance of hyaline coagulated mass, intense change in the bronchi and bronchioles, and little fibrinous exudation, are recorded by many investigators.[9] All the above changes were also observed in snuffles pneumonia caused by Bact. lepisepticum except no hyaline coagulated masses, as described in influenza, were observed, and perivascular infiltration was more often found in snuffles.

A more interesting contrast is the affection of the sinuses. McCartney and Olitsky [10] observed sinusitis in practically 100% snuffles rabbits and I also found it in 76% of such animals, while Fraenkel [11] observed sinusitis in 75% of influenza cases and Prym [12] also found sphenoid sinusitis in 77% of his influenzas.

DISCUSSION

In both spontaneous and experimental snuffles pneumonia caused by Bact. lepisepticum, pneumonia is the characteristic change. Perivascular edema and leukocytic infiltration, besides the ordinary forms of bronchopneumonia and hemorrhage, are observed, but these are not recorded in influenzal pneumonia in man [3,4,5] or in experimental B. influenzae pneumonia [6] except in Oguchi's [8] report. According to Cecil and Blake's report [7,13] in pneumococcus or streptococcus pneumonia, perivascular edema and infiltration, besides interstitial pneumonia and confluent lobar pneumonia, are often observed; and the bacteria penetrate the bronchial mucous membrane almost at once and spread rapidly through the perivascular lymph space in the alveolar walls to all parts of the lobes. In the influenzal pneumonia the bacteria are present in large numbers in the bronchioles and to less extent in the adjacent alveoli. Influenza bacilli, however, are rarely found in any part of the interstitial tissue, and, as MacCallum [4] has pointed out, the lymphatics are noticeably free from involvement. Therefore, B. influenzae in both the experimental and spontaneous disease seems to travel down the bronchi into bronchioles and subsequently involves the neighboring alveoli by contiguity.

Oguchi [8] has said that "B. influenzae is found not only in the portion in which there is leukocytal infiltration, serous exudate and hemorrhage, but also it is present in the interstitial tissue or alveolar walls which

[9] Fujinami, Matsubara: J. Assn. Jap. Int. Med., 1921, 9, p. 1. Fukshi, Naga Yama: Ibid. Katsnki: Ibid. Winternitz, Wason and McNamara: The Pathology of Influenza, 1920.

[10] J. Exper. Med., 1923, 38, p. 591.

[11] Virchows Arch. f. path. Anat., 1896, 143, p. 42.

[12] Deutsch. med. Wchnschr., 1919, 45, p. 880.

[13] J. Exper. Med., 1920, 32, p. 401.

show no noticeable change. At this time B. influenzae is found in small numbers on the inner side of alveolar walls or in the alveoli, but it is present numerously also in the lymph spaces of the alveolar walls or septa. From these results it may be considered that B. influenzae can enter directly into the alveoli; also it may be transferred continuously through the lymph spaces in the alveolar septum or alveolar walls and then invade cell groups on the surface."

According to the study of snuffles pneumonia reported in this paper, it is noticed that there is present perivascular edema and infiltration as in streptococcus or pneumococcus pneumonia, with bronchopneumonia as in influenzal pneumonia. And the bacteria can always be isolated from the blood, while in influenza the organisms rarely invade the blood stream. Also Bact. lepisepticum was isolated in pure culture from the purulent contents of the bronchi or bronchioles, or the exudate of the affected portion, and there were observed, microscopically, many phagocytes containing small bacilli in the stained films from the above materials. This bacterium is observed not only in the bronchioles and alveoli, but in the interstitial tissue, capillaries and lymph spaces. Smith [14] described these bacteria as creeping along the lymphatics, invading the serous cavities, and finally producing death.

From these observations it may be said that Bact. lepisepticum in snuffles pneumonia is distributed in two ways: through lymphatics; and directly from the bronchi into the bronchioles, then involving the neighboring alveoli by contiguity, a situation similar to that described by Oguchi [8] for B. influenzae. This may explain why snuffles pneumonia shows a greater tendency towards confluence than does B. influenzae pneumonia.

MacCallum [4] found that suppurative bronchiolitis with peribronchial pneumonia may be caused in some instances by streptococci. Fujinami and Matsubara [9] consider that there may be a close relation between the presence of B. influenzae and bronchiolitis, peribronchitis and bronchopneumonia, especially at the onset of a tendency toward suppurative inflammation. But the suppurative process in the snuffles pneumonia may apparently be due to Bact. lepisepticum, because this organism was demonstrated in pure culture from the pus and it possesses the ability to cause abscess formation, as described in a previous paper.

B. bipolaris, possessing a biological nature similar to B. lepisepticum, has often been demonstrated in cattle, swine, sheep, rabbits, buffalo, foxes, etc., and these organisms are considered the essential agent of

[14] J. Comp. Med. and Surg., 1887, 8, p. 24.

septicemia in these animals. On the other hand, B. bronchisepticus also is found in dog, cat, rabbit, guinea-pig, monkey, ferret, rat, pig and man.[15] Mallory [16] reported that in his experimental tests with whooping cough, he observed lesions similar to those caused by B. bronchisepticus in rabbits. Torrey and Rahe, as cited by Hoskins and Stout,[15] observed changes due to B. bronchisepticus similar to those of B. pertussis observed in dog and rabbit.

Therefore, in experimental tests with B. influenzae or B. pertussis on laboratory animals, we must always bear in mind that there are similar organisms present which produce changes very like those that are characteristic of these bacteria, and consider them a possible source of error in the results.

SUMMARY

The principal pathologic changes in snuffles caused by either Bact. lepisepticum or B. bronchisepticus in rabbits are rhinitis, sinusitis, and, in the lung, intense engorgement, hemorrhage and edema, bronchitis, bronchopneumonia, perivascular infiltration, inflammation of the serous cavities.

B. lepisepticum snuffles also causes perivascular edema, bronchiectasis, congestion of the inner organs and bacteremia.

B. bronchisepticus snuffles also causes peribronchial infiltration and bronchiolitis.

The pathologic changes in rabbits having snuffles induced experimentally by intranasal inoculation with secretion of snuffles containing Bact. lepisepticum or B. bronchisepticus and by injections of pure cultures of these organisms are similar in all respects to those of the naturally acquired diseases.

The pathologic changes in snuffles pneumonia caused by Bact. lepisepticum or B. bronchisepticus are similar in many respects, with the following differences. Frequent occurrence of abscesses, suppurative inflammation, bronchopneumonia, purulent pleurisy and marked lymphangitis are most prominent in the former, while hemorrhage and edema are most marked in the latter.

The pathologic changes in Bact. lepisepticum pneumonia seem to be nearly identical with experimental B. influenzae pneumonia in rabbits, and also very similar to influenzal pneumonia of monkeys, except perivascular infiltration and suppurative inflammation are more marked in the Bact. lepisepticum pneumonia.

[15] Hoskins, H. P., and Stout, A. L.: Jour. Lab. and Clin. Med., 1919, 5, p. 307.
[16] Boston M. & S. J., 1913, 169, p. 378.

The characteristic changes of human influenzal pneumonia are also observed to be closely similar to Bact. lepisepticum pneumonia in rabbits, except that in the human disease no hyaline coagulated masses are found and perivascular infiltration is less commonly observed.

In pneumonia, Bact. lepisepticum may invade the lungs in two ways—either through the lymphatics or through the respiratory passages. Suppurative processes in Bact. lepisepticum pneumonia are probably caused by Bact. lepisepticum.

In experimental tests on laboratory animals with B. influenzae or B. pertussis, the possible presence of Bact. lepisepticum or B. bronchisepticus in normal and infected animals must always be considered.

A COMPARATIVE STUDY OF PASTEURELLA CULTURES FROM DIFFERENT ANIMALS

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Bacteria of the hemorrhagic septicemia group, isolated from different animals and named B. bovisepticus, B. suisepticus, etc., have certain morphological and cultural characteristics in common, and to some extent similar pathogenic properties.

As early as 1886 it was shown by Hueppe [1] that the hemorrhagic septicemia organisms, although isolated from various sources, possess similar biological properties. They were given the name "Pasteurella" by Trevisan in 1887. Smith [2] recognized the close resemblance of B. bovisepticus and B. suisepticus. Baumgarten [3] reported that the agents of rabbit, swine, and cattle septicemia and of fowl cholera were probably identical. Ostertag [4] asserted that B. bovisepticus and B. avisepticus were identical with the bacteria causing swine plague and chicken cholera, respectively. Schirop [5] reported that strains of B. vitulisepticus isolated from pneumonia of calves, possessed fermentation reactions similar to those of B. avisepticus and B. suisepticus. Magnusson [6] described some strains from reindeer and showed them to be culturally similar to B. bovisepticus and B. avisepticus. Mori [7] observed that avisepticus immune serum agglutinated the strains derived from other animals than fowls. Gochenour [8] reported that a strain obtained from buffalo tissue could not be differentiated from hemorrhagic septicemia strains isolated from other domestic animals by either cultural, serological, or animal protection experiments. Gallagher [9] stated that rabbits immunized with a B. avisepticus strain possess a considerable degree of immunity to B. bovisepticus and that they also became resistant to infection with B. suisepticus.

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[1] Berl. klin. Wchnschr., 1886, 18, p. 753.
[2] Report Bureau of Animal Industry 12, 13, 1895-6, p. 149; J. Exper. Med., 1921, 33, p. 441.
[3] Lehrbuch der pathol. Mykologie II, p. 749.
[4] Ztschr. f. Infekt. d. Haust., 1908, 4, p. 1.
[5] Centralbl. f. Bakteriol., I, O., 1908, 47, p. 307.
[6] Ztschr. Infekt. d. Haust., 1914, 15, p. 61.
[7] Extract Exper. Sta. Record, 1918, 38, p. 887.
[8] J. Am. Vet. M. Ass'n., 1924, 65, p. 433.
[9] Ibid., 1916-7, 50, p. 708.

Bollinger [10] and others demonstrated the transmissibility and pathogenicity for other animals, of materials from animals which had died of "Wild- und Rinderseuche." Pease [11] reported that an infection in Indian buffalo was transmissible to other animals and that the isolated strains were identical with those causing "Rinderseuche." Beck and Koske [12] produced an infection in birds by feeding them with B. suisepticus cultures. Similar records are reported by many other investigators.

The reports mentioned indicate that the hemorrhagic septicemia organisms are identical or that they, at least, possess similar biological characters. There are, on the other hand, also many reports which indicate the existence of sub-groups or types. Voges and Proskauer [13] grouped them into four types according to their cultural characteristics. Jones [14] divided them into three distinct groups on the basis of their fermentation reactions on carbohydrates. Plasaj and Pribram [15] found three morphologically different types, namely atrichial, monotrichial, and oligatrichial rods. Wassermann and Ostertag,[16] Chamberland and Jouan,[17] Hutyra and Marek,[18] and Hutyra [19] reported that an immune serum produced with one strain furnishes active protection against the homologous strain but the protective action fails or only partially succeeds against other strains.

McGowan and Wang,[20] Busson,[21] Plasaj and Pribram,[15] and Jones [14] observed a varied serological relationship among the septicemia bacteria. Roderick [22] noted similarity between the antigenic properties of B. bovisepticus and B. suisepticus, while B. avisepticus and B. ovisepticus induced a somewhat different immunological response.

It was reported that buffalo septicemia rarely attacks cattle on the buffalo and cattle farms, but no sheep were infected with the "Wild-und Rinderseuche" (Kitt, Van Eecke,[23]); and fowl cholera, which is often pathogenic for chickens and other birds, rarely attacks water birds (Cornil and Toupet [24]). Kitt [25] was

[10] Wild- und Rinderseuche in der Umgebung von München, 1878.
[11] The Veterinarian, 1898, 71, p. 278.
[12] Arb. a. d. k. Gsndhtsamte., 1905, 22, p. 429.
[13] Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 20.
[14] J. Am. Vet. M. Ass'n., 1921, 60, p. 271.
[15] Centralbl. f. Bakteriol., I, O., 1921, 87, p. 1.
[16] Ztschr. f. Hyg. u. Infektionskrankh., 1904, 47, p. 416.
[17] Ann. de l'Inst. Pasteur, 1906, 20, p. 81.
[18] Special pathology and therapeutics of the diseases of domestic animals, 1912, p. 137.
[19] Kolle and Wassermann: Handbuch d. path. Mikroorganismen, 1913, 6, p. 77.
[20] J. Path. and Bact., 1915, 20, p. 21.
[21] Centralbl. f. Bakteriol., I, O., 1921, 86, p. 101.
[22] J. Infect. Dis., 1922, 31, p. 313.
[23] Tier-ärztl. Centralbl. f. Niederland, Indien, 1891, 5, p. 290.
[24] Compt. rend. Acad. d. sc., 1888, 106, p. 1747.
[25] Kolle and Wassermann: Handbuch d. path. Mikroorganismen, 1913, 6, p. 37.

unable to produce disease in the larger domestic animals by feeding them cultures of fowl cholera bacteria.

SOURCES OF CULTURES

(a) The Pathological Division of the Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

Pasteurella lepisepticum W_1 and W_2; bovisepticus 67 and 298; suisepticus Swine 1, no. 1 and Swine 2, no. 2.

(b) Dr. Theobald Smith, Rockefeller Institute for Medical Research, Department of Animal Pathology, Princeton, N. J.

B. bovisepticus I, isolated from cow with pneumonia; bovisepticus III isolated from lung of calf; rabbit septicemia R11, isolated from rabbit; avisepticus 8041 received from N. Y. Veterinary College.

(c) Dr. W. A. Hagan, N. Y. State Veterinary College, Department of Pathology and Bacteriology, Ithaca, N. Y.

B. avisepticus 1132, 1130, 1264, 1172, isolated from birds; bovisepticus 8260, 8318, 8102, 8128, isolated from typical and atypical outbreaks of cattle disease; ovisepticus C-35 from Colorado; and the buffalo septicemia.

(d) Dr. W. G. Sackett, Laboratory of Bacteriology, Agricultural Experiment Station, Fort Collins, Colo.

B. ovisepticus 33, isolated from heart blood of sheep.

(e) Department of Animal Husbandry, Animal Pathology Laboratory, University of Illinois, Champaign, Ill.

B. suisepticus 113, isolated from heart blood of rabbit injected with mucosa of large bowel of a pig; suisepticus 649 from vital organs of a rabbit injected with heart, liver, kidney and spleen emulsion of a pig.

(f) Bact. lepisepticum 99, 9, K, isolated by the writer in this laboratory from the heart blood of a rabbit with snuffles.

A total number of 26 strains were examined—lepisepticum, 6 strains; ovisepticus, 2; buffalo, 1; bovisepticus, 8; suisepticus, 4; avisepticus, 5.

All these 26 hemorrhagic septicemia organisms—from rabbits, cattle, sheep, swine, buffalo and guinea pigs, chickens and other birds—were found to be very small, slender, gram-negative, aerobic, non-hemophilic rods showing bipolar staining. They did not form spores nor did they produce gas in sugar media. No strain liquefied gelatin and only one —the buffalo strain—coagulated milk.

Attempts to differentiate the organisms of this group on the basis of their biochemical reactions were made with media and methods as follows:

(a) Brom cresol purple milk.

(b) Indol production. Ehrlich's para-dimethyl amidobenzaldehyde method,[26] using Armour's peptone.

(c) Nitrate reduction. Standard method.[27]

(d) Sugar fermentation. The medium used was sugar-free veal infusion broth, to which 0.5% carbohydrate was added. Brom cresol purple was used as indicator.

The initial hydrogen ion concentration of the media was P_H 7.4 to 7.5. All cultures were incubated at 37 C. for one week. The results obtained are shown in table 1.

[26] Berl. med. Wchnschr., 1901, 1, p. 151.

[27] Manual of Methods for Pure Culture Study of Bacteria, Am. Soc. Bact., 1923, p. 7.

The results show a striking similarity in most of the cultural characteristics of the strains studied. They agree in that they produce indol, do not show visible growth on potato, reduce nitrates to nitrites, and ferment some of the sugars. There was a slight variation in the fermentation reactions on lactose, maltose, salicin and trehalose. The experi-

TABLE 1

REACTION OF HEMORRHAGIC SEPTICEMIA CULTURES ON VARIOUS MEDIA

Culture Media	Lepisepticum 6		Bovisepticus 8			Ovisepticus 2	Avisepticus 5		Suisepticus 4	Buffalo Strain 1
	W_1	R11	8128	67	298	33	1172	1264	113	
		9	8102	I	III	C-35	1130	1132	649	
	W_2	99	8318					8041	1	
		K	8260						2	
Dextrose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Levulose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Mannite	+	+	+	+	+	+	+	+	+	+
Saccharose	+	+	+	+	+	+	+	+	+	+
Sorbit	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+
Lactose	0	0	∓	0	0	0	0	0	0	+
Maltose	0	∓	∓	∓	0	∓	∓	∓	0	+
Salicin	0	+	+	+	0	+	0	+	..	+
Trehalose	0	+	+	+	0	+	0	+	..	+
Adonite	0	0	0	0	0	0	0	0	0	0
Dextrin	0	0	0	0	0	0	0	0	0	0
Dulcite	0	0	0	0	0	0	0	0	0	0
Insulin	0	0	0	0	0	0	0	0	0	0
Raffinose	0	0	0	0	0	0	0	0	0	0
Rhamnose	0	0	0	0	0	0	0	0	0	0
Erythrit	0	0	0	0	0	0	0	0	0	0
Indol	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Milk	0	0	0	0	0	0	0	0	0	0 c
Potato	No visible growth									

Milk: 0 indicates no change in color, c indicates coagulation. ∓ indicates fermented sugar after 3-5 days' incubation.

ence of other workers is substantiated, however, for as a rule dextrose and saccharose are fermented while lactose is not. Gas formation does not occur with any of the sugars. Fitch and Nelson [28] found likewise the action of these organisms on carbohydrates to be quite constant. With one exception, there was no visible change in milk. The morphological and cultural characteristics agree with the observations of Smith [29]

[28] J. Am. Vet. M. Ass'n., 1923, 63, p. 147.
[29] J. Comp. Med. and Surg., 1887, 7, p. 24

and with De Kruif's [30] description of Bact. lepisepticum. It must be concluded from this work that it is impossible to differentiate the various organisms belonging to this group on the basis of their biochemical reaction.

The hydrogen ion concentration of dextrose, saccharose, lactose, and maltose cultures was determined daily during seven days. Sugar free veal infusion broth was used as a base to which 1.0% of carbohydrate was added. The initial hydrogen ion concentration was 7.4-7.5. Incubation took place at 37 C. The colorimetric method with phenol red and brom cresol purple as indicators was employed. It was noted that all cultures reached their final concentration after from 3 to 4 days.

The results are shown in table 2.

TABLE 2

HYDROGEN ION CONCENTRATION IN HEMORRHAGIC SEPTICEMIA CULTURES IN SUGAR MEDIA

Culture	Dextrose	Saccharose	Lactose	Maltose
Lepisepticum				
W1	6.0	6.1	7.6	7.6
R11, 9, 99	5.6-5.8	5.8-6.0	7.6-7.8	6.4-6.8
Bovisepticus				
67, 298	5.4-5.8	5.8-6.0	7.8	6.4 (67), 7.6 (298)
8128, 8102	5.4-5.6	5.4-5.6	6.2-6.8	6.4
Avisepticus				
1172, 1130, 1132	5.4-5.8	5.8-6.0	7.8	6.4-6.6
Ovisepticus				
33, 35	5.4-5.6	5.4	7.6	6.4
Suisepticus				
113, 649	5.6-5.8	5.6-5.8	7.8	7.6

SEROLOGIC TESTS

The method of producing antiserum was similar to that followed by other workers. Rabbits were injected subcutaneously with 18-20 hour cultures of the organisms suspended in 0.85% salt solution. A series of three daily injections was given followed by a rest period of from 4 to 5 days after which the injections were resumed. This procedure was carried out for a period of several weeks before a serum of sufficiently high titer was obtained. Heat-killed bacteria were used for the first of these injections and the dosage of the living culture was increased up to 10-15 mg. of organisms. Bleedings were made on the 10th to 11th day after the last injection, the blood being taken from the heart or the common carotid artery. The serum was drawn after the

[30] J. Exper. Med., 1921, 33, p. 773.

blood had clotted and was preserved in the refrigerator after the addition of a sufficient quantity of phenol to make a 0.5% solution.

The agglutination titer of the serums (table 3) shows that only serums of low titer were secured. This substantiates further the opinion that these organisms are poor antigens although their invasive power, particularly if recently isolated, is high.

TABLE 3

HIGHEST DILUTIONS OF ANTISERUMS GIVING POSITIVE AGGLUTINATION

Antiserum	Culture Number	Dilution
Bact. lepisepticum	9	640
Bact. lepisepticum	9 (sheep)	640
Bact. lepisepticum	W	320
B. bovisepticus	67	640
B. bovisepticus	298	320
B. avisepticus	1132	640
B. avisepticus	1130	400
B. ovisepticus	33	640

TABLE 4

AGGLUTINATION OF HEMORRHAGIC SEPTICEMIA STRAINS AND ANTISERUMS

Strain	Antiserums of Indicated Known Titer			
	Lepisepticum 9 1:640	Bovisepticus 67 1:640	Avisepticus 1132 1:640	Ovisepticus 33 1:640
Bact. lepisepticum R11, 9, 99 W_1, W_2, K	320–640 536	80–320 226	80–320 200	160–320 240
B. bovisepticus 67, 298, 8128 8102, 8318, 8260 I, III	40–320 100	80–640 450	40–640 130	40–640 155
B. avisepticus 1172, 1132, 1130 1260, 8041	80–320 176	80–640 192	160–640 480	80–160 112
B. ovisepticus 33, C-35	160	80–160 120	160–320 240	640
B. suisepticus 113, 649 1, 2	40–160 90	160–320 200	80–160 100	80–160 140
Buffalo strain	160	160	80	80

Agglutination.—Bacterial suspensions were prepared according to Dreyer's method.[31] The mixture of bacterial suspension and antiserum was incubated at 40-43 C. in a water bath over night, then read and recorded. Normal rabbit serum in a 1 : 20-40 dilution and serum-free bacterial suspensions were used as controls. The results are shown in table 4. Where agglutination occurred, the minimum, maximum and average dilutions of the antiserums are given.

[31] Cited in Stitt, Practical Bacteriology, Blood Work and Animal Parasitology, 1923, p. 243.

The results do not indicate that it is possible, by the methods used, to distinguish strains of these organisms from one another or to suggest a host specificity assuming that inherent differences may possibly occur.

Complement Fixation.—Complement fixation tests were carried out using the same strains and antisera. The serums were inactivated by heating at 56 C. for 30 minutes and the hemolytic and anticomplementary values determined previous to making each test, using the standard complement fixation. The antigens were prepared by Melick's method [32] with slight modifications. Young cultures of the organisms to be tested, grown on blood agar at 37 C. for 20 to 24 hours, were suspended in distilled water. After heating these suspensions in a water bath at 56 C. for 30 minutes, they were subjected to rapid alternations of freezing and thawing. This procedure was repeated from 5 to 8 times. The suspensions were mixed with 0.85% salt solution and preserved in the refrigerator. They were centrifuged and the clear supernatant fluid was used as antigen. The anticomplementary value of original suspension was between 0.005 to 0.0015 cc. of original suspension. One half of the anticomplementary value was used. A 5% suspension of well washed sheep red blood cells with two units of complement and two units of hemolytic amboceptor constituted the hemolytic system. The total volume in each tube was made up to 2.5 cc. The preliminary incubation for fixation of the complement was carried on at 37 C. in a water bath for one hour with occasional shaking, as was also that for the determinations of hemolysis. Readings were made immediately after incubation and again the following morning after 15 to 20 hours' storage in the refrigerator. The results substantiate those obtained with the agglutination reaction and therefore failed to demonstrate specific immunological characteristics in the cultures studied.

SUMMARY

Hemorrhagic septicemia bacteria isolated from various animals show a marked similarity in their biochemic, cultural and morphological characteristics. This limited systematic classification which finds acceptance among workers in this country differs somewhat from that advanced in Europe.

No evidence was found, by means of the agglutination and complement fixation reactions, of indicated specific host differences among these organisms, although it is quite possible that group differences may occur.

[32] J. M. Res., 1922, 43, p. 405.

The invasive power of these organisms is usually high, particularly when freshly isolated and especially for certain animals, notably the rabbit. Their antigenic power is low as measured by the immunity stimulated in the animal and by immunological reactions in vitro.

STUDIES UPON BACTERIAL SPORES

2. INCREASING RESISTANCE TO HEAT THROUGH SELECTION

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The fact of variation in resistance to heat among the individual spores of a given bacterial culture has been well established by the work of numerous investigators. This matter is of fundamental importance, particularly as it is concerned with food preservation, and it is essential that as much as possible be learned of the reasons for this variation and the factors influencing it.

That the physiology of bacteria may be markedly affected by differing cultural practices has long been known and evidence is rapidly accumulating that profound morphological changes may be brought about by careful cultural control. It is but a step, therefore, to the conception that modifications in spore resistance may be effected by modifications in cultural practices, and that possibly more highly resistant strains may be derived through a process of selection. In an earlier paper [1] the writer has set forth the results of experiments made to determine the relation of age and the environmental factors of humidity and temperature to bacterial spore resistance, which show that contrary to the commonly accepted idea that spores once formed have a fixed resistance, in reality the resistance varies with age and with the conditions under which the spores exist. In the present paper it will be shown that the resistance to heat of the spores within a given strain may be very greatly increased by a process of selection.

THE ORGANISM

The organism selected for the present study was Bacillus mycoides a culture of which was derived from the strain used in the experiments previously mentioned. The spores of this organism had shown in December 1923, when thermal death time tests were first made upon them, a resistance requiring an exposure of 2 minutes at 100 C. to overcome when suspended in distilled water and sealed within hard glass capillary tubes. Their resistance during the period of 9 months in which they were studied had been observed to increase four-fold.

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[1] J. Bact., 1926, in press.

In November of 1924 further resistance tests were made upon spores of this same strain and the previous findings confirmed. It was from spores surviving 7 minutes of exposure at 100 C. in these latter tests that the culture for the present study was secured.

METHODS OF PROCEDURE

The experiments were carried out according to the following plan:

A nutrient broth culture derived from selected survivors of thermal resistance tests was stored at ordinary room temperature for several weeks, or until the thick tenacious pellicle had sufficiently aged to make possible a reasonably even suspension of the spores by agitation of the tube. About 2 cc. of this suspension was then transferred to a tube of fresh nutrient broth. This, after shaking, was placed in a small water bath, the bath brought to boil over the bunsen flame and the boiling continued for 2 minutes. This was done to destroy any vegetative forms still remaining so that the cultures later used would be from freshly germinated spores. After this preliminary treatment the suspension was removed from the bath and one or more sand cultures prepared from it according to the method earlier described.[1] Clay-top petri dishes were used and just enough of the suspension was introduced to saturate the sand. These cultures were then placed in the 50% humidity chamber and incubated for 10 days at the temperature of 30 C.

At the end of this period spore suspensions were prepared by shaking a quantity of the spore-bearing sand in sterile distilled water and the spores washed free of metabolic products, culture residues, etc., by repeated centrifuging and resuspension in sterile distilled water. After being thrown out of suspension for the second time they were redistributed in fresh sterile water which was just sufficient in amount to give the desired concentration of spores. Suitable quantities of this suspension were then sealed into capillary tubes prepared from "Pyrex" glass and the thermal death time tests performed by exposure for measured periods of time in a constant temperature oil bath maintained at 100 C. Five tubes were used in each exposure.

The effect of the treatment was determined in all cases by inoculation of tubes of standard nutrient broth with the entire capillary tube and its contents, after the ends had been clipped off with a sterile instrument, and incubation at 30 C. In recording the results care was taken to show the number of days required for growth to appear in the tubes in order to check up on possible delayed germination Controls were kept to make certain the detection of failure in technic at any stage in the experiments.

EXPERIMENTAL FINDINGS

In the work herein reported two distinct series of experiments were carried on, which for convenience have been designated as series A and B. The cultures used in each were derived from the same original source, but in succeeding experiments were kept entirely distinct though receiving like treatment. For the sake of economy of space the tabular material will be confined, for the most part, to data from one series, though the results of the second will be discussed briefly at each step.

The first series of tests in this study was made on February 24, 1925. As in previous work the minimum period of exposure was ½ minute, the time increased by ½ minute steps up to 2 minutes and then by 1 minute stages beyond this point to a maximum of 9 minutes. This was judged as a sufficient range to cover any likely variations in resistance over previous findings. Table 1 shows the results of this first experiment.

It is seen that the resistance exceeded expectations, as all but one tube in the entire series showed viable spores. A slight tendency toward delayed germination is noted in the spores receiving the longer exposure, requiring in some cases six, and in one case seven, days to produce growth.

TABLE 1

THERMAL RESISTANCE OF SPORES OF BACILLUS MYCOIDES

Series A_1

Length of Exposure, Minutes	Growth from Treated Spores after Period in Days				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
½	2	2	2	2	2
1	2	2	2	2	2
1½	2	2	2	2	2
2	2	2	2	2	2
3	2	2	2	2	2
4	2	3	3	3	4
5	3	3	3	3	4
6	2	2	3	3	6
7	3	3	4	6	Lost
8	4	6	6	7	0
9	4*	4	6	—	—

Spores were from a culture developing from spores that survived an exposure of 7 minutes at the temperature of 100 C. in tests of Nov. 26, 1924.
Sand culture made Feb. 16, 1925.
Date of tests, Feb. 24, 1925.
Temperature of oil bath, 100 C.
A dash (—) indicates no test, and a small circle (0) no growth.
Controls remained negative.
* This culture selected as source of spores for series A_2.

In series B_1 made upon spores from sand culture prepared February 24 and tested on March 7, positive germination was secured in every instance even though the range of exposures was increased to 12 minutes. The controls remained negative. Germination in the tubes of these tests occurred in from 2 days in the case of the spores treated for 1 and 2 minutes to from 10 to 18 days in the case of those treated for 12 minutes. One culture developing from the spores surviving 12 minutes of exposure at 100 C. and germinating in 10 days was selected as the source for spores for the tests of series B_2.

In these two series of tests, therefore, a positive increase in resistance over any previously observed was demonstrated. How much greater

resistance might have been shown had longer exposures been made is not known. Curves based upon these and succeeding findings, however, indicate that the death time would have been found at a considerably higher point.

The second stage in the process of selection was begun by using the culture derived from spores that survived 9 minutes of exposure in the series A_1 tests of February 24. The sand culture was prepared from this on March 13, stored in the 50% humidity chamber at 30 C. for 10 days and the resistance tests made on March 23. Hoping to establish definitely the thermal death time in this case the range of the exposure periods was increased to 20 minutes. The results of these tests are presented in table 2.

TABLE 2

THERMAL RESISTANCE OF SPORES OF BACILLUS MYCOIDES

Series A_2

Length of Exposure, Minutes	Growth from Treated Spores after Period in Days				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
1	1	1	1	1	1
2	1	1	2	2	2
3	1	2	2	2	2
4	2	2	2	2	3
5	2	2	2	2	2
6	2	2	2	2	3
7	2	2	2	2	3
8	2	2	2	3	3
9	2	3	3	4	10
10	2	3	4	4	4
11	3	3	4	5	6
12	4	4	4	4	7
13	3	3	4	5	8
14	2	3	4	4	5
15	3	4	4	4	5
16	4	4	5	8	8
17	4	5	7	9	10
18	8	9	10	12	0
19	3	3	5	9	10
20	5*	7	10	11	0

Spores were from culture developing from spores (A_1) that withstood 9 minutes of exposure at 100 C. on Feb. 24, 1925.

Sand culture prepared March 13 and stored in 50% humidity chamber at 30 C. for 10 days. Tests made March 23, 1925. Temperature of oil both, 100 C.

Controls negative on April 21 when cultures were discarded.

* This culture selected as source of spores for series A_3.

Here again the increase in resistance through selection exceeded expectations, all but two of the tubes showing growth in from 1 to 12 days. A tendency toward delayed germination is again noted, those spores receiving the longest treatment in general requiring longer to show growth. Some variation in this respect is shown, however. In the case of the spores receiving 9 minutes of exposure one tube required 10 days to show germination, while two tubes from spores receiving 19 minutes of exposure showed germination in 3 days.

In the light of these results series B_2 tests were performed. The culture in this series of tests was derived from spores surviving 12 minutes of exposure at 100 C. on March 7. The sand culture was prepared on April 3, stored in the 50% humidity chamber at 30 C. for 10 days and the tests upon the spores carried out on April 13. The range of the exposure periods extended from 5 minutes as a minimum to 40 minutes. From 5 to 30 minutes the periods were increased by 1 minute and beyond the 30-minute point the increase was by 2-minute

TABLE 3

THERMAL RESISTANCE OF SPORES OF BACILLUS MYCOIDES

Series B_2

Length of Exposure, Minutes	Growth from Treated Spores after Period in Days				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
5	1	1	1	1	2
6	1	1	1	2	2
7	1	2	2	2	2
8	2	2	2	2	3
9	2	2	2	2	2
10	1	1	2	2	3
11	2	2	2	3	5
12	2	3	3	3	3
13	2	2	3	3	4
14	2	2	2	2	0
15	2	2	3	5	0
16	2	3	3	3	11
17	2	4	5	7	0
18	3	3	4	7	11
19	3	4	4	7	7
20	5	7	15	0	0
21	0	0	0	0	0
22	1	1	1	2	2
23	2	0	0	0	0
24	7*	11	14	16	0
25	0	0	0	0	0
26	0	0	0	0	0
27	17	0	0	0	0
28	0	0	0	0	0
29	0	0	0	0	0
30	4	0	0	0	0
32 to 40	0	0	0	0	0

Spores were from culture developing from spores that withstood 12 minutes of exposure at 100 C. on March 7, 1925.

Sand culture prepared April 3, 1925 and stored at 30 C. for 10 days in the 50% humidity chamber. Tests made April 13, 1925. Temperature of oil bath, 100 C.

Controls negative on May 4 when cultures were discarded.

* This culture reserved as source of material for series B_3.

additions. The results of this experiment bring out points not shown before in the present work and a table of them is given herewith:

In this experiment the range of the exposure periods was sufficient to show the high point in the stage of increased resistance. So far as series A_2 correspond with these in respect to exposure periods the results are entirely comparable, and it is probable that had a greater range been covered in the former, thermal death time figures closely approximating these would have been secured.

As the high point in resistance is approached irregularities in the matter of positive and negative growth in the culture tubes appear, due apparently to the fact that the great majority of the spores are rather easily destroyed and the suspension of more resistant viable spores is accordingly more dilute. Negative cultures under these circumstances are to be expected, and doubtless would show up more frequently where more dilute suspensions were used in resistance tests. The relative infrequency of the so-called "skips" in the data here presented is believed to be due to the fact that heavy suspensions were used.

TABLE 4

THERMAL RESISTANCE OF SPORES OF BACILLUS MYCOIDES

Series A_3

Length of Exposure, Minutes	Growth from Treated Spores after Period in Days				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
10	1	1	1	2	—
11	1	1	1	1	1
12	1	1	1	2	2
13	2	4	5	5	6
14	2	2	3	5	5
15	2	2	2	4	0
16	2	4	4	6	7
17	2	5	6	7	0
18	4	7	7	8	14
19	3	4	5	7	7
20	5	5	6	9	0
21	2	3	7	8	0
22	4	8	0	0	0
23	6	14	0	0	0
24	4	7	0	0	0
25	3	4	8	14	14
26	7	0	0	0	0
27	15	0	0	0	0
28	9*	0	0	0	0
29 to 40	0	0	0	0	0

Spores were from a culture developing from spores that withstood exposure to a temperature of 100 C. for 20 minutes on March 23, 1925.

Sand culture prepared April 11, 1925 and stored in 50% humidity chamber at 30 C. for 10 days. Tests made April 21, 1925. Temperature of oil bath, 100 C.

Controls remained negative until May 11, when all cultures were discarded.

* This culture selected for series A_4 tests.

Comparing the results of these two experiments with those preceding it is seen that a marked increase in resistance in the strain of Bacillus mycoides was secured.

The next step in the process of selection for resistance was taken with the culture derived from spores surviving an exposure of 20 minutes at 100 C. in the series A_2 tests of March 23. The sand culture in this case was prepared on April 11, stored in the humidity chamber as usual and the resistance tests made on April 21. The findings for series A_3 are given in table 4.

The data of table 4 show a continued heightening of resistance in the spores.

The results obtained in series B_3 showed a complete killing out of all spores at above 30 minutes of exposure. The figures were entirely similar to those shown in table 4 except that in two instances 56 days were required for growth to appear in the culture tubes, and in two others an indefinite period, determined by the limits of 64 and 102 days, were required for germination to occur. A culture arising from spores that survived 29 minutes of exposure at 100 C. and germinated in 4 days was reserved for the B_4 tests.

TABLE 5

THERMAL RESISTANCE OF SPORES OF BACILLUS MYCOIDES
Series A_4

Length of Exposure, Minutes	Growth from Treated Spores after Period in Days				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
15	2	2	3	3	3
16	2	3	4	4	5
17	3	3	4	—	—
18	2	2	4	4	4
19	2	3	3	3	3
20	2	2	2	4	5
21	2	3	3	4	—
22	2	3	3	4	5
23	2	2	3	4	4
24	2	3	3	4	5
25	4	4	4	4	4
26	3	3	4	5	—
27	2	2	2	4	4
28	2	3	3	3	4
29	2	3	3	4	8
30	3	3	3	3	4
32	4	4	4	4	6
34	3	4	4	4	8
36	3*	3	5	7	7

Spores were from a culture developing from spores that withstood 28 minutes of exposure at 100 C. on April 21, 1925.
Sand culture prepared on June 16, 1925 and stored in 50% humidity chamber at 30 C. for 10 days. Tests made June 26, 1925. Temperature of oil both, 100 C.
Controls were negative when discarded on July 13, 1925.
* This culture reserved for series A_5 resistance tests.

The fourth stage of the investigation was entered when resistance tests were performed upon the spores derived from the culture obtained from spores surviving 28 minutes of exposure at 100 C. in the series A_3 tests of April 21. The experimental findings of series A_4 tests are presented in table 5.

Here again the increase in resistance exceeded expectations, every culture tube showing growth in from 2 to 8 days.

The B_4 series were then performed using as the source of spores a culture reserved from the B_3 tests of June 15. It will be remembered that these spores had indicated a thermal death time of 31 minutes at

100 C. The sand culture was made on July 13, stored as usual and the tests made on July 23. The range of exposure periods extended from 20 minutes as the minimum to 44 minutes as the maximum.

This increase in the severity of the treatment proved insufficient for the determination of the thermal death time as most of the culture tubes prepared from the treated spores showed growth for all exposure periods in from 3 to 26 days. A few others showed positive germination after somewhat longer incubation. The controls remained negative at the end of 64 days when they were discarded.

It is seen, therefore, that the spores that had shown a thermal death time of 31 minutes in the case of the B_3 culture, and of 29 minutes in

TABLE 6

THERMAL RESISTANCE OF SPORES OF BACILLUS MYCOIDES
Series A_5

Length of Exposure, Minutes	Growth from Treated Spores after Period in Days				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
20	5	6	8	11	50
21	11	50	50	50	50
22	4	6	7	11	50
23	3	50	50	50	50
24	50	50	50	50	0
25	4	50	50	50	0
26	50	50	50	50	0
28	11	50	50	50	0
30	50	50	50	0	—
32	8	50	50	50	0
34	50	50	50	50	50
36	50	50	50	0	—
38	50	50	50	0	0
40	50	50	50	0	0
42	50	50	50	0	0
44	50	50	50	50	50
46	50	50	50	0	0
48	50	50	50	50	0
50	50	50	50	50	50

Spores were from a culture developing from spores that survived the temperature of 100 C. for 36 minutes in the tests of June 26, 1925.

Sand culture prepared on July 27, 1925 and stored in 50% humidity chamber. Tests made on Aug. 6, 1925. Temperature of the oil bath, 100 C.

Controls remained negative up to time the experiment was terminated on Sept. 25, 1925.

the case of the A_3 culture had given rise to spores showing a resistance in excess of 36 minutes in the case of the A_4 culture and in excess of 44 minutes in the case of the B_4 culture.

With the culture reserved from the survivors of the A_4 tests made on June 26 the fifth group of experiments was undertaken on August 6. The range of the exposure periods was increased to 50 minutes. Shortly after the middle of August, and before the results of the experiment could be determined entirely it became necessary to be absent from the laboratory for an extended period and the final readings could not be

made until September 25, or 50 days after the tests were performed. The tubes showing the reading of 50 in the following table represent those tubes in which the spores germinated after August 18 and before September 25. The details of the experiment are given in table 6.

The data speak for themselves.

It was expected to check the results of these last tests by similar ones upon the culture reserved for the B_5 series, but pressure of other work prevented this from being done and forced the abandonment of the present study at this point. It is impossible, therefore, to say how far this increase in resistance might have been carried by repeated selections and cultivation of surviving resistant spores. The results

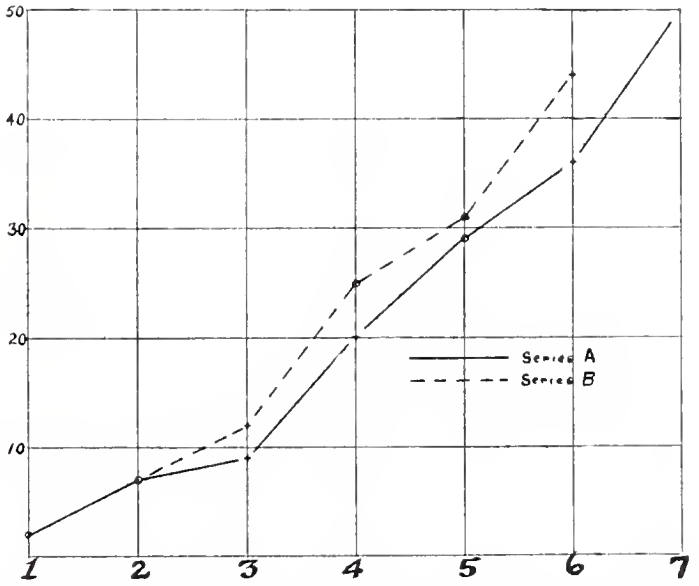

Chart 1.—Showing the increase in thermal death times for the spores of Bacillus mycoides through selection. Plus signs indicate that the death time was above these points. Points within circles indicate definite death time readings. The abscissa figures indicate: 1, original culture; 2, after 9 months' earlier cultivation; 3, first selection; 4, 5, 6, 7, the 2nd, 3rd, 4th and 5th selection, respectively.

secured appear sufficiently striking and clear cut to warrant their presentation.

To show through just what range the resistance of the spores of the strain of Bacillus mycoides has passed during the period they have been under observation in this laboratory the following chart has been prepared. The points of the curves are based upon the data presented in the tables and discussion preceding. The plus signs (+) show where the spores under observation exceeded in their resistance the points indicated on the chart, and the points within circles (⊙) mark the thermal death times as established in the tests.

DISCUSSION

The results of this study emphasize again the variable character of bacterial spores within a given strain. That some few individuals in a given suspension are much more resistant than the great majority of their fellows is abundantly shown, and in this respect the present study confirms the observations of the most recent workers in this field.

Delayed germination, which has been studied by Eckelmann,[2] Burke,[3, 4] Dickson and his associates [5] and Burke, Sprague and Barnes,[6] has likewise been noted here. Germination periods varying from 1 to over 64 days have been observed within the same strain. "Heat inhibition," thought at first by Burke [3] and by Weiss [7] to be the explanation for this phenomenon, apparently is not an important factor here, for long delayed germination in tests where the exposure period was short, and prompt germination of spores subjected to more rigorous treatment in the same series of tests have been observed. This indicates normal inherent differences in the spores themselves as the cause of delayed germination rather than heat effects. In the more recent papers of Burke [4] and of Dickson and his associates [8] this interpretation is given to delayed germination findings.

Irregularity in the development of subcultures from treated spores and breaks in the continuity of positive cultures, particularly as the thermal death time is approached, support the findings of Esty and Meyer,[9] Esty and Williams [10] and Dickson and his associates [8] as well as our own previous observations.[1] The interpretation of results of this sort has been given by these workers as being due to the presence within a given suspension of certain spores which are much more resistant to heat than most of the spores present, their numbers being so few that occasionally a culture will be prepared in which none of them are present. This seems to be the logical conclusion to be derived from these findings.

Since the completion of this work the attention of the writer has been drawn to a brief statement of the results of similar experiments by Bigelow and Esty.[11] While these workers did not consider their

[2] Centralbl. f. Bakteriol., 2 Abt., 1917, 48, p. 140.
[3] J. Am. M. A., 1919, 72, p. 88.
[4] J. Infect. Dis., 1923, 33, p. 274.
[5] J. Am. M. A., 1922, 79, p. 1239.
[6] J. Infect. Dis., 1925, 36, p. 555.
[7] Ibid., 1921, 28, p. 70.
[8] Ibid., 1925, 36, p. 472.
[9] Ibid., 1922, 31, p. 650.
[10] Ibid., 1924, 34, p. 516.
[11] Ibid., 1920, 27, p. 602.

results of sufficient significance, apparently, to warrant detailed consideration in their paper, it is of interest to note that the nature of their findings in so far as they are set forth, is in complete accord with those here presented.

These findings upset our inherited notions and teachings that the thermal death relations of spores from a given bacterial strain are constant, but they are by no means at variance with the results of bacteriological work having to do with the attenuation and the enhancement of virulence among pathogenic microbial forms.

Preliminary studies only have been made to determine the basis for these differences in thermal resistance among spores and the evidence in hand is insufficient in amount to warrant definite conclusions. More information upon this important subject is greatly needed, for until more is known of the reasons for these differences satisfactory and reliable methods of spore destruction cannot be developed, and the technology of food preservation will continue to be subject to the laws of chance.

SUMMARY

Evidence is presented which shows that some bacterial spores derived from the resistant survivors in thermal death time tests possess higher resistance to heat than the original spores; and that by a process of selection a strain of bacteria may be obtained whose spores attain a resistance of at least twenty-five times that of the original spores.

THE BACTERICIDAL ACTION OF ACRIDINE DYES AND THE ADJUVANT EFFECT OF SERUM

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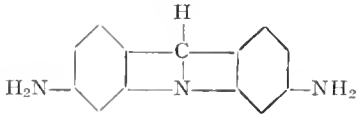
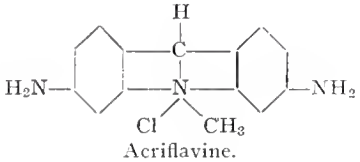
The dye known as acriflavine in England and America, and as trypaflavin in Germany, was prepared by Benda [1] for Ehrlich in 1912. It is the methyl chloride addition compound of 3, 6 diamino acridine (now known as proflavine).



Proflavine.



Acriflavine.

Ehrlich and Benda employed acriflavine in curing trypanosome infections in mice. Shiga [2] first used this dye as a bactericide. He showed that a dilution of 1-250,000 killed cholera germs in three hours.

Browning and Gilmour,[3] working with proflavine, showed that staphylococcus, anthrax, and typhoid were killed by high dilutions of this dye. They also reported the remarkable fact that it is more potent in serum than in peptone water. Browning and his collaborators [4] soon enlarged these observations. A 1-20,000 dilution of the dye was found to kill staphylococcus in peptone water after 24 hours at 37½ C. The titer in inactivated ox serum was 1-200,000, a ten-fold increase in potency. This is a unique phenomenon among disinfectants, whose activity is usually greatly depressed by proteins. As chemotherapeutic agents of necessity work in the presence of serum, such a property should be of the highest value. Furthermore, it was stated that proflavine and acriflavine are less toxic to tissue cells than to bacteria, and that they are practically non-irritating to the tissues. On injecting large doses of proflavine intravenously into rabbits, Browning and Gulbransen [5] showed that the rabbit's serum became definitely germicidal. The same authors [6] showed that mice could be saved from a lethal dose of pneumococci, but only if the dye was given within 15 minutes after the infecting dose.

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[1] Ber. d. deutsch. chem. Gesellsch., 1912, 45, p. 1787.
[2] Ztschr. f. Immunitatsforsch. u. exper. Therap., 1913, 18, p. 65.
[3] J. Path. & Bact., 1913, 18, p. 144.
[4] Brit. M. J., 1917, 1, p. 73; 2, p. 70.
[5] Proc. Roy. Soc., London, 1918, 90B, p. 136.
[6] J. Path. & Bact., 1919, 22, p. 256.

Since then, a considerable number of investigators have reported on the use and action of acriflavine, some disputing, some confirming the observations of Browning and his collaborators. The adverse evidence will be examined first.

Dakin and Dunham [7] pointed out that an infected surface or cavity did not contain serum alone; cells of various kinds and their lytic products were always present. These reduce the germicidal action of the dye. Hence they proposed defibrinated blood or serum mixed with fresh muscle juice as a more rational test fluid than serum alone. In both of these media, Dakin and Dunham found the germicidal titer for flavine (proflavine) for staphylococcus to be 1-3,000 instead of 1-200,000 as given by Browning.[4]

Fleming [8] showed that flavine in a 1-2,000 dilution immediately and completely stopped the emigration of leukocytes. Browning, he pointed out, exposed leukocytes to flavine for 20 minutes to 2 hours, whereas he exposed bacteria for 24 hours. Fleming exposed leukocytes for 5 hours to a 1-8,000 concentration of flavine, after which they failed to phagocyte cocci. He stated that leukocytes, pus cells, necrotic tissue, and even fresh granulation tissue have a strong affinity for the dye, which is thus removed from solution and rendered ineffective. These observations were corroborated in large part by Hewlett.[9]

Tissue necrosis due to acriflavine treatment of wounds has been observed by Drummond and McNee,[10] Bashford, Hartley, and Morrison,[11] Pearson,[12] Pilcher and Hull,[13] and Bauman.[14] Morgan [15] found that the movement of leukocytes was very much inhibited by acriflavine in a dilution of 1-64,000, and Mueller [16] showed that the toxicity of both acriflavine and proflavine was as great for tissue cultures as for streptococci.

Acriflavine treatment of experimentally infected animals was undertaken, with negative results, by Tubby,[17] Gay and Morrison,[18] Beckwith,[19] Spencer,[20] and Meleney and Zau.[21]

[7] Brit. M. J., 1917, 2, p. 641.
[8] Lancet, 1917, 2, pp. 341, 508.
[9] Ibid., p. 493.
[10] Ibid., p. 640.
[11] Brit. M. J., 1917, 2, p. 849.
[12] Lancet, 1918, 1, p. 370.
[13] Brit. M. J., 1918, 1, p. 172.
[14] München. med. Wchnschr., 1923, pp. 731, 775.
[15] Lancet, 1918, 1, p. 256.
[16] J. Path. & Bact., 1919, 22, p. 640.
[17] Lancet, 1919, 2, p. 838.
[18] J. Infect. Dis., 1921, 28, p. 1.
[19] Ibid., 29, p. 494.
[20] J. Lab. & Clin. Med., 1924, 9, p. 322.
[21] J. Am. M. A., 1925, 84, p. 337.

On the other hand, numerous reports of more successful results with the acridine dyes have been made. Various observers (Drummond and McNee,[10] Neufeld and Schieman,[22] Gay and Morrison,[18] and Gay and Beckwith [23]) have found the acriflavine titer to be distinctly higher in serum than in broth. Gay and Morrison [18] found that phagocytosis of streptococci was not inhibted in the least by an acriflavine concentration of 1-1,600, while one twentieth of this concentration sufficed to kill the streptococci in the presence of leukocytes. Acriflavine was found to be as active in pus (streptococcus empyema fluid from rabbits) as in serum.

Neufeld and Schieman [22] and Neufeld, Schieman and Baumgarten [23a] were able to save some mice infected with pneumococci; somewhat better results being obtained with proflavine than with acriflavine. Feiler,[24] Rienhardt [25] and Browning and Gulbransen [26] obtained excellent results in treating diphtheria infected wounds with acriflavine.

A large number of clinical investigators have also reported favorably on the use of acriflavine or proflavine in infections. They have been used in war wounds by Drummond and McNee,[10] Carslaw and Templeton,[27] and Pilcher and Hull.[13] Furstenau [28] and Horvath and Jano [29] have used them in eye infections. Davis and Harrel,[30] and Baer and Klein [31] have found irrigations with acriflavine of great value in treating gonorrhea. Davis [32] found oral administration of acriflavine useful in sterilizing the urine. Bohland [33] recommended intravenous administration of acriflavine in influenza, pneumonia, pyelitis, septicemia, and articular rheumatism, and Strecker and Willey [34] have used the dye with favorable results in patients showing the sequelae of encephalitis.

Morgenroth, Schnitzer and Rosenberg [35] have recently introduced "Rivanol," another acridine derivative, as a chemotherapeutic agent against the streptococcus. This substance, like acriflavine and proflavine,

[22] Deutsch. med. Wchnschr., 1919, 45, p. 844.
[23] Am. J. Hyg., 1922, 2, p. 267.
[23a] Deutsch. med. Wchnschr., 1920, 46, p. 1013.
[24] Ztschr. f. Immunitatsforsch. u. exper. Therap., 1920, 30, p. 950.
[25] Ztschr. f. Hyg., 1922, 95, p. 1.
[26] Brit. M. J., 1925, 1, p. 688.
[27] Lancet, 1918, 1, p. 634.
[28] Ztschr. f. Augenheilk., 1918, 40, p. 1.
[29] Jahrsversammlung d. Ungar. Ophthalm. Gesellsch., Budapest, 1923.
[30] J. Urol., 1918, 2, p. 257.
[31] München. med. Wchnschr., 1918, p. 970.
[32] J. Urol., 1921, 5, p. 215; 1924, 11, p. 29.
[33] Deutsch. med. Wchnschr., 1919, 45, p. 797.
[34] Am. J. Psychiat., 1925, 4, p. 631.
[35] Deutsch. med. Wchnschr., 1921, 47, p. 1317.

is stated to be more active in serum than in broth. The high therapeutic value claimed for this substance is ascribed to the fact that even non-germicidal concentrations rapidly reduce the virulence of the streptococcus.[36]

In this paper, a re-investigation of the action of acriflavine and proflavine is presented. The first part deals with the activity of acriflavine in vitro, and is directed chiefly to an explanation of the remarkable adjuvant action of the serum. It was hoped also by these test tube experiments to determine the optimal conditions for the chemotherapeutic use of the dye in the animal body. The second part deals with an attempt to cure streptococcus empyema in rabbits by the use of acriflavine and proflavine.

TECHNIC OF IN VITRO EXPERIMENTS

Acriflavine (Heyl), obtained from the National Aniline and Chemical Co., was used. Serial dilutions of the dye were prepared in distilled water; these were kept in the ice box in the dark while not in use. Under these conditions the acriflavine solutions kept for weeks without change. For the tests, 0.1 cc. quantities of these dilutions were measured into small test tubes, and 0.9 cc. of the test fluid (broth, serum, pus, etc.) then added. The bacteria were added to the test fluid and well mixed before this was added to the acriflavine. The tubes were then placed in a water bath at 37½ C.; at various intervals, a loopful from each tube was streaked on the surface of blood agar (streptococcus) or plain agar (staphylococcus and typhoid bacillus). The contents of the tubes were always stirred with the platinum loop before the subculture was made.

The Streptococcus pyogenes used in these experiments was the "H" strain, passage culture, used by Gay and Stone,[37] Gay and Morrison,[18] Foster,[38] Gay and Beckwith,[23] Gay and Rhodes[39] and Gay and Clark.[40] This is a hemolytic streptococcus that is highly virulent for rabbits. At the beginning of these experiments it had already been passed through 75 rabbits; an animal passage was made on the average of once a month. The pus from a rabbit dead of the infection (empyema) was kept in the ice box, where the organism remained alive and virulent; to obtain a broth culture, 0.1 cc. of the pus was inoculated into a tube of plain infusion broth. Growth in this medium is vigorous and diffuse. On plating out such a broth culture, the colony count was usually 100 million colonies per cc. In these experiments, each cc. of the test fluid was inoculated with 0.1 cc. of a 24 hour broth culture of the streptococcus.

The Staphylococcus aureus used chiefly was an old laboratory stock culture. As a check, some of the experiments with staphylococcus were repeated with four recently isolated staphylococci—two from furuncle and two from osteomyelitis. The recently isolated cultures showed no important differences from the stock

[36] Morgenroth, Schnitzer, and Berger: Klin. Wchnschr., 1923, 2, p. 1633. Schnitzer and Amster: Ztschr. f. Hyg., 1924, 102, p. 287.

[37] J. Infect. Dis., 1920, 26, p. 265.

[38] J. Bact., 1921, 6, p. 161.

[39] J. Infect. Dis., 1921, 29, p. 417; 1922, 31, p. 101.

[40] Ibid., 1925, 36, p. 233.

culture. The standard inoculum with this organism was 0.01 cc. of a 24 hour broth culture in each cc. of test fluid.

The typhoid bacillus used was the "Pfeiffer" strain. The amount of inoculum was the same as for the staphylococcus.

Care was taken to adhere strictly to the standard dosages given above, as the germicidal titer of acriflavine varies considerably with the size of the inoculum.[7, 23]

As germicidal tests are very commonly carried on in the presence of culture media, the first experiments were directed to determining the effect of the different constituents of culture media on the germicidal titer of acriflavine.

TABLE 1

THE GERMICIDAL TITER OF ACRIFLAVINE IN MEDIUMS OF DIFFERENT COMPOSITION, ALL AT PH 7.4

	Staphylococcus			Streptococcus			Typhoid		
Mediums	2 Hours	4 Hours	24 Hours	2 Hours	4 Hours	24 Hours	2 Hours	4 Hours	24 Hours
M/20 sodium phosphate..	1: 20,000	1: 40,000	1: 80,000	1: 10,000	1: 20,000	1: 160,000	1: 20,000	1: 40,000	1: 80,000
Merck's peptone, 1%.....	.....	.....	1: 40,000	.....	.....	.....	.....	.....	1: 20,000
Difco peptone, 1%.....	.....	.....	1: 80,000	.....	.....	.....	.....	.....	1: 40,000
Parke Davis peptone, 1%	1: 20,000	1: 40,000	1: 80,000	1: 5,000	1: 20,000	1: 160,000	1: 10,000	1: 20,000	1: 40,000
Beef infusion.....	1: 10,000	1: 10,000	1: 40,000	1: 2,500	1: 10,000	1: 80,000	1: 5,000	1: 10,000	1: 20,000
Beef infusion broth.....	1: 10,000	1: 10,000	1: 40,000	1: 2,500	1: 10,000	1: 80,000	1: 5,000	1: 10,000	1: 20,000
Gelatin, 1%.....	1: 5,000	1: 10,000	1: 80,000	1: 2,500	1: 5,000	1: 80,000	1: 10,000	1: 20,000	1: 40,000

Table 1 compares the effect of sodium phosphate solution, three different brands of peptone, beef infusion (without peptone), beef infusion broth (with 1% Difco peptone), and 1% gelatin. The most striking fact brought out is the consistent lowering of the acriflavine titer by media containing meat infusion. Kligler[41] found that meat extract broth gave lower titers than peptone water with such germicides as beta napthol and salts of hexamethyllenetetramine, and Graham Smith[42] found that meat infusion lowered the titer of acriflavine and homoflavine. As seen in table 1, Merck's peptone gives lower titers than do the other two brands. While gelatin in a 1% solution gives the same 24 hour titer as do Difco and Parke Davis peptone, the 2 and 4 hour titers are very much less (except for typhoid). Gelatin in a 3% solution was found by Gay and Beckwith[23] to reduce the 24 hour acriflavine titer from 1-25,000 (peptone water control) to 1-10,000 when the typhoid bacillus was the test organism.

As sodium or potassium phosphate added to test fluids is very useful as a buffer for stabilizing the P_H, a more detailed investigation was made of their effect on the acriflavine titer. In concentrations between 0.1% and 0.5%, these salts when added to peptone water, beef infusion broth, or 1% gelatin, made no difference whatsoever in the acriflavine titer when the P_H was brought back to 7.4 after the addition of the phosphate.

[41] J. Exper. Med., 1918, 27, p. 463.

[42] J. Hyg., 1919, 18, p. 1.

TABLE 2

THE EFFECT OF THE PH ON THE 24 HOUR GERMICIDAL TITER OF ACRIFLAVINE

P_H	Staphylococcus	Streptococcus	Typhoid
6.2	1: 20,000	1: 40,000	1: 12,000
6.6	1: 20,000	1: 40,000	1: 12,000
6.8	1: 40,000	1: 40,000	1: 24,000
7.1	1: 40,000	1: 40,000	1: 24,000
7.4	1: 40,000	1: 80,000	1: 48,000
7.7	1: 40,000	1: 80,000	1: 48,000
8.2	1: 80,000	1:160,000	1: 96,000
8.6	1:320,000	1:320,000	1:192,000

The mediums for the staphylococcus and typhoid determinations consisted of 1% peptone water (Parke Davis) with 0.5% di-sodium phosphate. The streptococcus tests were done in infusion broth containing the same amount of phosphate.

Table 2 shows the striking effect of the P_H of the medium on the germicidal effect of the acriflavine. On the acid side of P_H 7.7, the effect of small changes of P_H is not very marked; but as the P_H becomes more alkaline, the titer rises very rapidly.

When the dye is added to nutrient agar and the bacteria are inoculated on the surface, the effect of the P_H is somewhat different, and is even more striking. In this case, the bacteriostatic effect of the dye in agar is measured. Table 3 gives the greatest dilution of acriflavine that inhibited the growth of bacteria on nutrient agar.

TABLE 3

INHIBITING CONCENTRATION OF ACRIFLAVINE IN NUTRIENT AGAR

P_H	Staphylococcus	Typhoid
6.6	1: 5,000	1: 5,000
7.0	1: 10,000	1: 10,000
7.4	1: 30,000	1: 30,000
7.8	1:120,000	1:120,000
8.2	1:240,000	1:240,000
8.6	1:480,000	1:480,000

The nutrient agar contained 1% peptone, 0.5% sodium phosphate, and 2% agar. Plates were incubated 48 hours before reading.

On comparing tables 2 and 3, an interesting difference is brought out. As was remarked before, there is no very striking rise in the acriflavine titer (for staphylococcus and streptococcus at least) between P_H 6.2 and 7.7 (table 2), while a sharp increase takes place on further addition of alkali. In table 3, however, the inhibiting titer is proportioned to the OH ion concentration throughout the P_H range tested.

A comparison of tables 2 and 3 also brings out a paradox that requires explanation. Thus, at P_H 7.0 (table 3) the staphylococcus multiplies in what seems a concentration of acriflavine that is actively germicidal (table 2). The reason for this is that the agar has a high affinity for the dye, rendering a large fraction of it ineffective. This can be easily demonstrated by slanting some agar in a tube, pouring an equal volume of dilute acriflavine over it, and incubating over night. Much more than half of the dye will enter the agar layer. This effect of agar on the acriflavine titer is similar to the effect of 3% gelatin and of gum acacia, as noted by Gay and Beckwith.[23] Graham Smith[42] also noted the effect of agar on the acriflavine titer, and showed that the inhibiting titer, within certain limits, varied inversely as the concentration of agar in the media.

That an alkaline reaction increases the germicidal activity of basic organic disinfectants was first shown by Prowazek.[43] Prowazek worked with a protozoan (Colpidium), and found that dilute alkali increased the toxic action of alkaloids and of three basic dyes—methylene blue, azure, and neutral red. He states that these dyes visibly penetrate more rapidly in alkaline than in acid or neutral solutions. Traube[44] found that the toxicity of methylene blue, toluidine blue, and methyl green were increased by dilute alkali. Braun and Schafer,[45] Kligler,[41] and Michaelis and Dernby[46] showed that the bactericidal activity of quinine derivatives and caffeine was increased by shifting the P_H to the alkaline side. Graham Smith[42] showed that the addition of alkali markedly increased the germicidal power of acridine dyes and of crystal violet.

Browning, Gulbransen, and Kennaway[47] give the following table for the germicidal titer of acriflavine for B. coli at different reactions:

PH of Peptone Water	Titer of Acriflavine
4.0 to 5.0	1- 2,000
6.0 to 7.0	1- 10,000
8.0 to 9.0	1- 40,000
11.0	1-200,000
Serum at PH 8.0	1-200,000

Davis and Harrell[30] and Davis and White[48] showed that the acriflavine titer in urine is 20 times greater at P_H 8.0 than at P_H 6.0 when B. coli is the test organism; only a slight difference was found, however, when the tests were made with staphylococcus. Beckwith[49] tested crystal violet in bile, salt solution, and serum; brilliant green and basic fuchsin in bile; and acriflavine in serum. In each case the germicidal titer was increased as the P_H became more alkaline. Fleischer and Amster[49] also reported increased germicidal action of acriflavine as the P_H became greater.

The reason for this remarkable effect of the P_H on the germicidal action of basic dyes is not yet known. The following explanations have been suggested:

1. The P_H of the medium effects primarily the bacterial cell, rather than the dye. This was first suggested by Kligler,[41] and again by Stearn and Stearn.[50] The latter authors point out that as the bacterial protein acids are very weak, their compounds with basic dyes tend to hydrolize; an alkaline P_H would prevent such hydrolysis.

2. The dye base may have a greater affinity for, or a greater penetrating power into cells than has the dye salt. This explanation rests chiefly on the work of Michaelis and Dernby[46] who showed that the germicidal activity of various quinine derivatives varied with the P_H exactly as does

[43] Archiv. f. Protistenk., 1910, 18, p. 221.
[44] Biochem. Ztschr., 1912, 42, p. 496.
[45] Berlin. klin. Wchnschr., 1917, vol. ??, p. 885.
[46] Ztschr. f. Immunitatsforsch. u. exper. Therap. O., 1922, 34, p. 194.
[47] J. Path. & Bact., 1919, 23, p. 106.
[48] J. Urol., 1918, 2, p. 299.
[49] Ztschr. f. Immunitatsforsch. u. exper. Therap. O., 1923, 37, p. 327.
[50] J. Bact., 1924, 9, p. 494.

the dissociation residue of a weak base, from which they concluded that only the undissociated base was active.

3. The alkali may cause a tautomeric rearrangement of the dye molecule, as occurs in many indicators. Fleischer and Amster [49] recall the readiness with which alterations of the P_H may cause the appearance or disappearance of double bonds in various dye molecules.

The next experiments were directed to observing the effect of the P_H on the acriflavine titer in serum.

TABLE 4

COMPARISON OF THE ACRIFLAVINE TITER IN BROTH AND SERUM

Medium	P_H	Staphylococcus	Streptococcus	Typhoid
Infusion broth	7.4	1: 40,000	1: 80,000	1: 40,000
Rabbit serum	7.4	1: 20,000	1: 80,000	1: 40,000
Rabbit serum (unadjusted	8.4	1: 80,000	1:320,000	1:160,000

Incubation with acriflavine was for 24 hours at 37.5 C. The rabbit serum was inactivated at 56 C. for 10 minutes.

The serum was brought to P_H 7.4 by adding 1.0 cc. of sterile N/5 KH_2PO_4 solution to every 10 cc. of serum. It was then incubated for 6 hours and adjusted to P_H 7.4. The P_H determinations were made by Cullen's method.

The P_H of fresh serum which is protected from loss of CO_2 is 7.4. As soon as CO_2 is given off, the P_H rapidly increases to 8.4 or 8.8. The rate of this change will vary with the temperature and the relative area of the free surface of the serum. The CO_2 is lost very rapidly at 56 C., the temperature used for inactivating the complement of serum. Serum stored in deep columns presents a relatively small free surface; in columns of 1 cm. or less in depth (as in ordinary germicidal tests) the free surface is relatively large, and the CO_2 escapes rapidly. These facts are in general well known to biochemists. They have been recently studied by Plotz and Schoen.[51]

If serum has become alkaline by loss of CO_2, it cannot be permanently restored to P_H 7.4 by merely adding acid until that point is reached. For the same process repeats itself: the CO_2 that was liberated from the serum bicarbonates is again slowly given off until nearly the same alkalinity is reached. Even a third and fourth neutralization will fail to establish the serum at P_H 7.4; this will not occur until all of the bicarbonate is exhausted. A solution of $NaHCO_3$ in water behaves in exactly the same way.

A rapid and satisfactory way of adjusting the P_H of the serum at 7.4 is as follows: to every 10 cc. of serum add 1 cc. of N/5 KH_2PO_4 solution (any other acid would do as well). Exhaust with a vacuum

[51] Ann. de l'Inst. Pasteur, 1924, 38, p. 923.

pump to draw off the CO_2, or incubate over night at 37 C. The P_H will be very near to 7.4, to which point it is now carefully adjusted. If it is desired to buffer the serum still more heavily, 1/10 volume of sterile M/2 phosphate solution at P_H 7.4 may be added.

In these experiments the P_H of protein containing fluids was determined by the method of Cullen.[52] The serum is diluted with 20 volumes of neutral isotonic salt solution to which indicator has been added. To prevent loss of CO_2, the surface is covered with oil or vaseline. The colorimetric reading at 20 C. plus a correction factor gives the actual P_H at 38 C. This correction factor varies somewhat with the species of animal from which the serum was obtained; for horse serum it is given by Cullen as + 0.16. Thus, if the apparent P_H of horse serum at 20 C. is 7.30, the actual P_H at 38 C. is 7.46. Cullen does not state what corrections are necessary for more acid or more alkaline ranges, or for other indicators than phenol red. In these experiments a correction of + 0.20 has been made for all colorimetric readings on serum and pus.

Table 4 shows that the P_H of unadjusted rabbit serum that has been heated to 56 C. for 10 minutes was 8.4. Only the unadjusted serum shows the remarkable increase in the acriflavine titer over the broth control. When the serum was permanently set at P_H 7.4, the titer for the streptococcus and typhoid bacillus was the same as for the broth control, while that for staphylococcus was less. It is clear that the adjuvant effect of unadjusted serum is due to its alkalinity.

There is another way in which this experiment can be done, without making any adjustment of the P_H whatever. This consists of preventing all escape of CO_2 during the course of the experiment. The blood, as soon as drawn, is placed in a sterile tube and corked; it is then centrifugated. The bacteria are added to the serum and mixed, taking care to prevent loss of CO_2 by unnecessary exposure. As the inoculated serum is measured into the test tubes (a hypodermic syringe of 5 or 10 cc. capacity is useful for this purpose), an assistant covers each column with a layer of sterile vaseline. An oil that is liquid at incubator temperature is worthless for this purpose. Protected with vaseline in this manner, the P_H remains constant at 7.4 for 24 hours in the incubator. Instead of vaseline, tightly fitting sterile corks were used with equally good results. In table 5 are shown the results of such an experiment. In this case, the serum was not heated to 56 C. to inactivate the complement.

Plotz and Schoen [51] have shown that serum sealed in glass ampules slowly becomes more acid. This change, however, is small in 24 hours, not exceeding 0.1 unit of the P_H scale. This is too small to cause any error in the final result.

[52] J. Biol. Chem., 1922, 52, p. 501.

Table 5 clearly confirms, by another method, the conclusion drawn from table 4, that the alkalinity of unadjusted and unprotected serum is the cause of its adjuvant effect.

TABLE 5

THE EFFECT OF THE PH ON THE GERMICIDAL TITER OF ACRIFLAVINE IN SERUM

Medium	PH	Streptococcus	Typhoid
Beef infusion broth	7.4	1: 80,000	1: 40,000
Human serum	7.4*	1: 80,000	1: 40,000
Human serum	8.6†	1:320,000	1:160,000

The tubes were incubated for 24 hours with the acriflavine. Each tube in the three series was tightly stoppered with a sterile cork.

* The PH of this series was maintained by preventing all loss of CO_2.

† The serum of this series was exposed to the air until all of the free CO_2 had been given off.

TABLE 6

THE EFFECT OF THE PH ON THE GERMICIDAL TITER OF ACRIFLAVINE FOR THE STAPHYLOCOCCUS, IN PLEURITIC FLUID AND IN PEPTONE WATER

PH	Human Pleuritic Fluid	Peptone Water
5.8	1:20,000	.....
6.1	1:20,000	1: 40,000
6.6	1:20,000	1: 40,000
7.4	1:20,000	1: 40,000
7.7	1:40,000	1: 80,000
8.6	1:80,000	1:160,000

The tubes were incubated for 24 hours.

In the experiment shown in table 6, the titer for staphylococcus in human pleuritic fluid is consistently lower than in the phosphate peptone water of the same P_H. There is no agreement at all between this experiment and the one previously quoted from Browning, Gulbransen and Kennaway,[47] in which the titer for B. coli in peptone water was found to be 1-40,000 at P_H 8.0, while the titer in serum at the same P_H was 1-200,000. On the other hand, this experiment agrees very well with the results obtained by Graham Smith [42] who brought both his serum and his peptone water to the neutral point for neutral red, and found the serum titer for staphylococcus slightly lower than the peptone water titer.

At one stage of this investigation, before the full importance of the hydrogen ion concentration was realized, an attempt was made to isolate from serum or ascitic fluid a hypothetical "adjuvant substance," to which the adjuvant properties of whole serum could be ascribed. The method used for fractionating the serum was, in part, that of Hardy and Gardiner.[53]

[53] J. Physiol., 1910, 40, p. 68.

To 1 part of serum or ascitic fluid at 0 C., 4 parts of acetone or freshly distilled alcohol at −8 C. were added. The precipitate was washed with ether and dissolved in sterile water (fraction 1). The filtrate was evaporated to a concentration of 50% acetone and then extracted with petroleum ether, thus removing the fats and cholesterol esters. A portion of this defatted filtrate was evaporated until all odor of acetone was gone (fraction 2). The remainder was acidified with H_2SO_4 and again extracted with petroleum ether; the petroleum ether layer was evaporated to dryness and the residue (the fatty acids of the serum soaps) taken up in dilute sodium hydroxide solution (fraction 3). The aqueous acetone layer was neutralized and evaporated down until all odor of acetone was gone (fraction 4). Each fraction was brought to P_H 7.4 and 1/10 volume of M/2 phosphate solution at P_H 7.4 added; then each fraction was brought to the volume of the equivalent amount of serum and heated to 56 C. for 30 minutes for sterilization.

TABLE 7

THE EFFECT OF ASCITIC FLUID FRACTIONS ON THE GERMICIDAL TITER OF ACRIFLAVINE WITH STAPHYLOCOCCUS, P_H 7.4

Test Fluid	24 Hour Germicidal Titer
M/20 sodium phosphate	1:40,000
Ascitic fluid, whole	1:20,000
Fraction 1 (proteins)	1:20,000
Fraction 2	1:80,000
Fraction 3 (soaps)	1:80,000
Fraction 4	1:40,000

The acetone and petroleum ether used in this experiment left no germicidal residue on evaporation.

Fractions 2 and 3, which contain the serum soaps, are in themselves germicidal to the streptococcus without the addition of acriflavine.

In table 7, the proper control fluid for fraction 3 is the sodium phosphate solution. Therefore the serum soaps, though themselves non-toxic for the staphylococcus have slightly increased the germicidal action of the acriflavine for this germ. This suggested the addition of various chemically pure soaps to fluids containing acriflavine.

TABLE 8

THE GERMICIDAL TITER OF SOAPS

Soap	Staphylococcus		Streptococcus		Typhoid, 24 Hours
	30 Minutes	24 Hours	30 Minutes	24 Hours	
Sodium oleate	0	0	1:16,000	1:16,000	0
Sodium laurate	1:200	1:1,000	1: 1,000	1: 4,000	<1:1,000
Sodium ricinolate	0	1: 200	1: 2,000	1: 4,000	<1:1,000
Sodium palmitate	0	0	0	1: 1,000	<1:1,000

All soap dilutions were made in phosphate infusion broth at P_H 7.4. The temperature was 37.5 C. 0 means that a 1:200 dilution did not sterilize.
< means "less than."

Certain of the soaps are in themselves highly germicidal for various species of bacteria (Lamar [54] and Walker [54a]). The action of the several soaps, without acriflavine, was tested on the three organisms here studied.

With a few exceptions, the results of table 8 agree with the findings of Walker.[54a] Sodium laurate was found toxic for all four strains of staphylococcus studied, whereas Walker found only one strain out of several that was killed by this soap. For the streptococcus, sodium oleate was found more germicidal, sodium laurate less than in Walker's experiments, which suggests that different strains of the same species may vary greatly in their susceptibility to soaps.

TABLE 9

THE ADJUVANT EFFECT OF SOAPS ON THE ACRIFLAVINE TITER

	Staphylococcus	Typhoid
Control broth	1: 40,000	1:40,000
Sodium oleate, 1:3,000	1: 80,000*	1:20,000
Sodium laurate, 1:10,000	1: 80,000	1:40,000
Sodium laurate, 1:3,000	1:320,000	1:40,000
Sodium ricinolate, 1:3,000	1:160,000	1:40,000
Sodium palmitate, 1:3,000	1: 40,000	1:80,000

All soap dilutions were made in phosphate infusion broth at P_H 7.4. The titers given are for the 24 hour period.

* This titer varied considerably in different experiments as explained below.

Table 9 shows that a soap, in a concentration that is not in itself germicidal, can yet considerably increase the titer of acriflavine. Thus, sodium laurate, in a concentration of 1-10,000, doubled the acriflavine titer for staphylococcus, and a concentration of 1-3,000 multiplied the titer by eight; while in table 8 it is seen that the germicidal concentration of sodium laurate alone for this organism is 1-1,600. Sodium ricinolate, itself only slightly germicidal, increased the acriflavine titer for staphylococcus four fold. With sodium oleate and staphylococcus, some very irregular results were obtained; in some experiments, a 1-3,000 dilution of this soap increased the acriflavine titer four fold, and in a few the titer was reduced to one half that of the control; in the majority of experiments, the results were as given in table 9.

With the typhoid bacillus, sodium oleate invariably lowered the acriflavine titer. Sodium laurate in a 1-3,000 concentration left the titer unchanged, as did sodium ricinolate. Sodium palmitate doubled the acriflavine titer in five experiments, and left it unchanged in one.

Because of their high initial toxicity for streptococci, different concentrations of soaps had to be chosen for work with this organism.

[54] J. Exper. Med., 1911, 13, p. 1.

[54a] J. Infect. Dis., 1924, 35, p. 557.

TABLE 10

THE ADJUVANT EFFECT OF SOAPS ON THE ACRIFLAVINE TITER WITH STREPTOCOCCUS

	Titer for Period of		
	1 Hour	2½ Hours	24 Hours
Broth control without soap	1: 5,000	1: 10,000	1: 80,000
Sodium oleate, 1:100,000	1: 5,000	1: 20,000	1: 80,000
Sodium oleate, 1:50,000	1: 20,000	1: 80,000	1: 80,000
Sodium oleate, 1:20,000	1:160,000	1:320,000	1:640,000
Sodium palmitate, 1:3,000	1: 5,000	1: 10,000	1: 80,000
Sodium laurate, 1:8,000	1: 5,000	1: 20,000	1:160,000

All dilutions were made in phosphate infusion broth at P_H 7.4. Controls without acriflavine all showed growth.

Table 10 shows that sodium oleate, even in extremely high dilutions, has a very marked adjuvant effect on the acriflavine titer for the streptococcus. This is apparent in the 1 hour and the 2½ hour titers even when the 24 hour titer is the same as in the control.

None of the soaps tested had any effect on the acriflavine titer in serum at P_H 7.4, even when employed in as high a concentration as 1-1,000 (higher concentrations were not tested). This inhibiting action of serum on soaps was noted by Lieberman,[55] Lieberman and Fenyvessy [56] and Lamar.[54] These investigators added boric acid to their serum soap mixtures to prevent this inhibiting action. Walker states that the presence of even 5% of serum reduces the killing strength of sodium oleate for streptococcus from N/640 to N/20. Table 11 gives the results of a test on the germicidal action of sodium oleate in a 1-1,000 solution in various dilutions of serum:

TABLE 11

THE EFFECT OF SERUM ON THE GERMICIDAL ACTION OF 1-1,000 SODIUM OLEATE ON THE STREPTOCOCCUS

	Time of Test			
Serum Dilution	1 Hour	2½ Hours	9 Hours	24 Hours
1: 2	+	+	+	+
1: 4	+	+	+	+
1: 8	+	+	+	+
1:16	+	+	+	—
1:32	+	+	—	—
1:64	—	—	—	—

The serum was adjusted to P_H 7.4 and diluted with infusion phosphate broth at the same P_H. + growth, — no growth on subculture.

While, as was stated before, the soaps in a dilution of 1-1,000 have no effect on the acriflavine titer in whole serum, the following experiment shows that they may exert a very marked adjuvant effect in diluted serum.

[55] Biochem. Ztschr., 1907, 4, p. 25.

[56] Ztschr. f. Immunitatsforsch. u. exper. Therap., 1909, 2, p. 436.

TABLE 12

THE EFFECT OF 1-1,000 SODIUM OLEATE ON THE ACRIFLAVINE TITER FOR STREPTOCOCCUS IN DILUTED SERUM

Time	Germicidal Acriflavine Titers			
	Serum 1:4 (Control)	Serum 1:4 with Oleate 1:1,000	Serum 1:8 (Control)	Serum 1:8 with Oleate 1:1,000
20 minutes	<1: 2,500	<1: 2,500	<1: 2,500	1: 2,500
30 minutes	<1: 2,500	<1: 2,500	<1: 2,500	1: 5,000
1 hour	<1: 2,500	1: 2,500	<1: 2,500	1:10,000
2½ hours	1: 2,500	1: 5,000	1: 2,500	1:20,000
6 hours	1:20,000	1:40,000	1:20,000	1:40,000
24 hours	1:80,000	1:80,000	1:80,000	1:80,000

Serum dilutions were made in phosphate infusion broth at P_H 7.4. < means "less than."

Table 12 shows that when serum is diluted 1-4, the addition of 1-1,000 sodium oleate doubles the efficiency of the acriflavine during the first 6 hours; after that the titer is the same as in the control. But when the serum is diluted 1-8, a very considerable increase in the germicidal action of the acriflavine is noted during the first few hours. That there is no direct germicidal action of the sodium oleate alone is shown by the fact that the serum soap and the serum control both ran the same 24 hour titers.

The value of soaps in augmenting the action of germicides has already been noted by Lamar [53] in connection with antipneumococcic serum, and by Davis and Schwartz [57] in connection with various silver preparations, chlorazene and organic mercurials. Berkeley and Bonney [58] recommended a proflavine oleate paste as a dressing in open wounds.

It was thought possible that the metabolic products of bacteria might have an effect on the acriflavine titer, so the following experiment was undertaken: Streptococcus was grown in serum broth for 24 hours, centrifugated, and the supernatant fluid adjusted to P_H 7.4. The sediment was resuspended in an equal volume of sterile broth and the culture supernatant and control serum broth were inoculated with it in the usual way. To allow for the incomplete removal of streptococci by centrifugation the inoculum into the culture supernatant was made slightly smaller than into the control; this was controlled by plating and colony counts.

The differences shown in table 14 are not large; in fact, in some experiments, no differences were found at all between the two series. But when it is remembered that the culture supernatant contains the

[57] J. Urol., 1920, 4, p. 409.
[58] Brit. M. J., 1919, 1, p. 152.

TABLE 13

THE EFFECT OF METABOLIC PRODUCTS ON THE ACRIFLAVINE TITER FOR STREPTOCOCCUS

	Time of Tests		
	2 Hours	6 Hours	24 Hours
Control serum broth	1:2,500	1:40,000	1:80,000
Culture supernatant	1:2,500	1:20,000	1:40,000

accumulated metabolic products of the streptococcus, and is, therefore, an unfavorable medium in itself, it makes the small differences found all the more significant. It would seem therefore, that the metabolic products of the streptococcus antagonize the action of the acriflavine. Similar experiments made with the staphylococcus were negative.

The next tests were done with streptococcic empyema fluid from experimental empyemas in rabbits. This material, obtained from different animals, varied considerably in the number of organisms and pus cells it contained, and in its P_H. Therefore it is not strange that the titer varied considerably with different samples of the empyema fluid, even though the organism was always the same.

TABLE 14

THE 24 HOUR ACRIFLAVINE TITER OF DIFFERENT SAMPLES OF EMPYEMA FLUID

Sample	Alkali Added	Titer	P_H	Remarks
A	.....	<1: 2,400	7.0	10 million colonies per cc.
B	.....	<1: 2,400	6.4	28 million colonies per cc.
B	0.6% Na_2HPO_4	1:11,000	7.5	
B	0.4% $NaHCO_3$	1:11,000	7.7	
B	.....	1:11,000	8.2	Left uncorked
C	.....	1: 4,800	6.2	15 million colonies per cc.
C	0.4% $NaHCO_3$	1: 4,800	7.2	
D	.....	1:11,000	6.2	
D	0.6% Na_2PO_4	1:11,000	7.5	
D	0.4% $NaHCO_3$	1:22,000	7.8	
E	.....	1: 2,800	7.4	
E	NaOH	1: 5,600	8.0	
F	.....	<1: 3,200	6.3	
F	NaOH	1: 3,200	8.6	
G	.....	1:16,000	7.1	
H	.....	<1: 2,800	7.0	
H	0.4% $NaHCO_3$	1: 2,800	8.2	
I	.....	<1: 3,200	6.4	

All tubes with the one exception noted were either layered with vaseline or tightly stoppered with sterile corks to prevent loss of CO_2. < means "less than."

The P_H of empyema fluid obtained from experimentally infected rabbits, was found to vary from 6.2 to 7.4. According to Schade, Neukirch, and Halpert,[59] the P_H of inflammatory exudates varies from 5.96 (acute furuncle) through 6.2 (empyema), 6.6 (chronic empyema)

[59] Ztschr. d. ges. exper. Med., 1921, 24, p. 11.

to 7.09 (chronic serous pleuritis). Four of the fluids given in table 15 (A, E, G, H) are more alkaline than the findings of Schade would lead us to expect. As animals often died during the night, it is possible that these four atypical findings were due to post mortem change. Schade showed also that pus, unlike serum, does not always reach the same P_H on losing CO_2 when exposed to the air. A very acid pus may have no alkali (bicarbonates) left; it will still be acid even when all the free CO_2 is gone. A pus of low acidity behaves like serum and will approach P_H 8.2 to 8.6 on exposure to the air.

In table 14, the P_H of the samples of empyema fluid was varied by adding sterile di-sodium phosphate or bicarbonate or dilute sodium hydroxide. In most instances the acriflavine titer was raised somewhat by so doing, but the effect of an alkaline P_H was found to be decidedly less than when the medium was broth or serum. Certainly, other factors seem to be more important than the P_H. These appear to be the pus cells, fibrin, and products of cellular autolysis that are found in variable amounts in these fluids. In table 1 it was pointed out that meat infusion lowers the titer of acriflavine; pus probably contains similar substances to those found in meat infusion. In table 14 it was shown that the metabolic products of this streptococcus probably lower the acriflavine titer. That the pus cells, fibrin, and tissue debris bind a great part of the acriflavine is obvious to the eye, and was noted by the earliest workers. Thus Fleming [8] added proflavine to pus, centrifuged it immediately, and determined the amount of dye free in the supernatant fluid. He found that ⅔ to ¾ of the dye was bound by the sediment.

Most workers have reported very unfavorably on the ability of acriflavine to sterilize pus. Fleming [8] was able to sterilize only one out of seven samples of pus from war wounds by a 1–1,000 concentration of proflavine. Hewlett [9] reports a concentration of 1–200 to 1–1,000 as necessary to sterilize pus, while Browning, Gulbransen, Kennaway, and Thornton [60] found a strength of 1–2,000 necessary to sterilize an empyema pus. On the other hand, Gay and Morrison [18] reported that a 1–200,000 concentration of acriflavine sterilized streptococcic rabbit empyema fluid; their titers in pus were practically the same as their titers in serum. This is 10 times the maximum titer here found (table 15), and fully 40 times the average titer. In reconciling the results obtained by Gay and Morrison with those recorded here, it is necessary to bear two factors in mind: (1) Gay and Morrison did not protect their empyema fluid from loss of CO_2, and hence probably worked with an alkaline fluid; and (2) though the same strain of streptococcus was used in both sets of experiments, the germ is now 100,000 times as virulent as it was when Gay and Morrison worked with it, and it is quite possible that the physical and chemical makeup of the empyema fluid has altered as well as the virulence of the organism calling it forth.

[60] Brit. M. J., 1917, 1, p. 73.

ANIMAL EXPERIMENTS WITH ACRIFLAVINE AND PROFLAVINE

The streptococcus used in these animal experiments was the "H" strain already described. It has now passed through the pleural cavities of 79 rabbits in the space of 5 years, and its virulence has increased enormously in that time. In 1920, Gay and Stone[37] found the intrapleural MLD to be 0.1 to 0.2 cc. of a 24 hour broth culture; there was little tendency for early extension into the opposite pleural cavity or the pericardium, and the blood culture was negative until just before death. At the present time, the intrapleural MLD of the same streptococcus is 0.000,001 cc. of a 24 hour culture.[40] Extension into the opposite pleural cavity occurs in 24 hours; soon after the pericardial cavity and the anterior mediastinum are infected, and the blood culture is usually positive in 48 hours. In spite of this, rabbits that have received as much as 1000 MLD will survive for 4 to 5 days after infection.

In these experiments, 0.001 to 0.000,1 cc. of a 24 hour broth culture in a volume of 1 cc. was injected into one pleural cavity. This corresponds to 1,000 to 100 lethal doses. Twenty to twenty-four hours later, treatment was begun. After this interval there are already a few cc. of exudate in the infected cavity, and the streptococci have proliferated enormously.

Gay and Morrison,[18] working with this same streptococcus, treated experimental empyema in rabbits by injecting acriflavine into the infected pleural cavities. While they were able in many cases to reduce greatly the numbers of living streptococci in the infected pleural cavities, they did not succeed in saving any animals. In continuing these experiments of Gay and Morrison, it was determined first to try the effect of weak alkali injected with the acriflavine to neutralize the acidosis and provide a more favorable P_H for the action of the acriflavine.

Exper. 1.—Rabbit no. 5, weight 1820 gm., received 0.001 cc. of a 24 hour broth culture in the right pleural cavity; 21 hours later, 4 cc. of a 1–400 solution of acriflavine in 2% $NaHCO_3$ were injected into the right pleural cavity. The same dose was repeated into the same cavity 3½ hours later. The rabbit died next day. At autopsy, 27 cc. of thin, lightly colored fluid were found in the right cavity. Its P_H was 7.0; the bacterial count was 2,200,000 colonies per cc. In the left pleural cavity were 8 cc. of fluid; the P_H was 7.1 and the bacterial count 200,000 colonies per cc.

Although this rabbit received alkali enough considerably to modify the local acidosis, and also received a large dose of acriflavine (11.1 mg. per kilo), sterilization was not effected. In fact, the rabbit died sooner than the untreated controls. On the basis of this and other similar experiments, it was felt that the toxic effect of the acriflavine outweighed any benefit derived from its germicidal action.

According to Browning and Gulbransen[5] the dye proflavine is 1/5 as toxic as acriflavine, yet is practically the equal of the latter as a germicide. They found that rabbits tolerate as much as 70 mg. per kilo intravenously, whereas Meleney and Zau[21] found a dose of 12½ mg. per kilo of acriflavine to be fatal to 50% of rabbits tested. Hence it was decided to continue the experiments with the much less toxic proflavine.

Exper. 2.—On March 5, rabbit no. 7, weight 1930 gm., was infected as before. Twenty-four hours later, a warmed mixture of 4 cc. of 0.5% proflavine in salt solution plus 5 cc. of 3% $NaHCO_3$ was injected into the infected cavity. Five hours later, another 4 cc. of 0.5% proflavine in salt solution was injected.

The rabbit was dead the next morning. In the right pleural cavity 8 cc. of fluid were found; the P_H was 6.0. In the left cavity, 5 cc. were found, with the same P_H. Streptococci were numerous in both fluids.

This animal received only 40 mg. of proflavine, less than 1/3 of the tolerated dose. In spite of this, the rabbit died sooner than the untreated controls. The infection, apparently, was not checked at all, as evidenced by the very acid P_H of the empyema fluid, even after treatment with alkali. It was thought that possibly the bicarbonate might be too alkaline and exert a harmful effect; but experiments without the bicarbonate turned out no better. It should be mentioned that the $NaHCO_3$ solution used was always freshly prepared and was not heated; its P_H was 8.4.

In the next experiment, sodium oleate was added to the proflavine solution to take advantage of the adjuvant effect of the oleate. As proflavine oleate is insoluble in water, it was found desirable to add 0.5% of gelatin to act as a protective colloid to hold the proflavine oleate from precipitating out. The gelatin was first freed from calcium by washing with dilute acetic acid in the cold.

Exper. 3.—Rabbit no. 9, weight 1900 gm., was inoculated with 0.001 cc. of 24 hour broth culture as before, on March 10. On March 11, the following solution was prepared:

2 cc. of 3% $NaHCO_3$.
2 cc. of 1% sodium oleate.
5 cc. of 0.5% proflavine in physiological salt solution.
1 cc. of 5% gelatin.

This solution was warmed to body temperature before injection. At 11:30 a. m., 23 hours after infection, 6 cc. of this fluid, containing 15 mg. of proflavine, were injected into the right pleural cavity, and 2 cc. into the left cavity as a prophylactic. At 12:40 p. m. this injection was repeated.

At 2:40 p. m., 7 cc. of highly colored, turbid fluid were withdrawn from the right pleural cavity. Its P_H was 6.7. On centrifuging out the cells, it was found that the proflavine concentration in the supernatant fluid was about 1–5,000. A portion of this fluid was immediately diluted with broth and 0.1 cc., 0.01 cc., and 0.001 cc. plated out. These cultures all remained sterile.

At 2:50 p. m. the same dosage (6 cc. into the right cavity, 2 cc. into the left) was repeated.

At 4:40 p. m., 13 cc. of highly colored fluid were withdrawn from the right pleural cavity. Cultures made as before also remained sterile. The dosage given before was repeated.

On March 12, a blood culture was made, drawing the blood from the femoral vein. It showed 50 colonies of streptococci per cc.

On March 13, the rabbit was dead. There were 4 cc. of fluid in the right pleural cavity, P_H 6.4; bacterial count, 400 colonies per cc. There were 8 cc. of fluid in the left cavity, P_H 6.0, with a count of 1,200,000 colonies per cc. There was pus in the anterior mediastinum, and the pericardium was infected. The subcutaneous tissues at the sites of the needle punctures on the right side were infected.

This rabbit received in all, 42 mg. of proflavine per kilo, well below the maximal tolerated dose. In addition, it received 18 mg. of sodium oleate per kilo. The right pleural cavity was unquestionably sterilized, but infection of the left

cavity and the pericardium, septicemia, and reinfection were not prevented. It is not likely that reinfection took place along the sites of the needle punctures. Every time the pleural cavity was entered, a new site, well away from the others, was chosen. If reinfection had occurred in this way, one would expect that the right cavity would be heavily infected at death, which was not the case. It seems more likely that reinfection took place via the anterior mediastinum, pericardium, and left cavity.

Other experiments were made along this general plan without striking improvements. By giving larger prophylactic doses into the left cavity, spread of infection to that side was prevented (rabbits no. 10 and 15). Giving proflavine intravenously as well as intrapleurally did not check the septicemia (rabbit no. 15). Rabbits no. 18 and 31 were treated with proflavine in 50% egg white instead of the oleate (Gay and Beckwith[23] found egg white germicidal to this streptococcus), but the results were distinctly inferior.

In the next experiments, several changes were made in the method. Di-sodium phosphate was used as alkali instead of sodium bicarbonate, as the latter did not seem to control the acidosis successfully. This may be because the carbonic acid-sodium bicarbonate system is not a good buffer around P_H 7.4. Instead of injecting rather strong solutions of proflavine, the pleural cavity was irrigated with a much weaker solution (1–4,000 to 1–5,000). This is more in harmony with clinical experience of the irritating effects of 1–1,000 solutions of acriflavine and proflavin.[10, 11, 12, 13, 32, 61]

Exper. 4.—On April 9, rabbit no. 20, weight 1950 gm., received 0.000,1 cc. of broth culture of streptococcus "H" in the right pleural cavity. On April 10, the following solution was prepared:

450 cc. of distilled water.
50 cc. of 5% gelatin solution.
3 gms. Na_2HPO_4.
3 gm. NaCl.
20 cc. of 4% sodium oleate.
0.125 gm. of proflavine.

This was sterilized by heating to 60 C. The fluid was in each case heated to body temperature before injecting into the pleural cavity. At 12:40 p. m., 24 hours after infection, the right pleural cavity was irrigated in the following manner: 20 cc. of the above fluid were injected and withdrawn in a few moments. This was repeated 5 times in the course of 40 mintues. Finally, 20 cc. of the mixture were left in the cavity and the animal released for 1 hour.

At 2:15 p. m., 22 cc. of turbid yellow fluid were aspirated. Its concentration of proflavine was 1–15,000 (colorimetric); its P_H was 7.7; its bacterial count was 620 colonies per cc.

At 2:20, 4:25, and 6:40 p. m., the above procedure was repeated. Aspiration fluids obtained at 6:40 and 8:40 were found to be sterile.

At 5:10, 20 cc. of proflavine oleate solution were injected into the left pleural cavity as a prophylactic.

On April 15 (6 days after infection) this rabbit appeared in good health and was eating normally. It was bled from the carotid and autopsied.

The blood culture was sterile. There were 27 cc. of fluid in the right cavity, of P_H 7.1. Culture of 0.01 cc. gave innumerable colonies. There was considerable fibrin deposit in the right cavity, but no adhesions. The left cavity contained 27 cc. of fluid, P_H 7.4. The colony count was 40 colonies per cc. The left lung and pleural cavity appeared normal, except for the fluid. The pericardial sac

[61] Bond, C. J.: Brit. M. J., 1917, 1, p. 6.

showed a light deposit of fibrin; a drop of the pericardial fluid gave 3 colonies of streptococci. The anterior mediastinum was normal and sterile. This rabbit had already lived one day longer than the average control when it was killed.

The results with this experiment were very much more favorable than any that were previously obtained. Reinfection had occurred, but the process was no longer acute.

Exper. 5.—Rabbit no. 22 received 0.000,05 cc. of a 24 hour broth culture in the left pleural cavity on April 22. The next day at 11 a. m., (21½ hours after infection) the left pleural cavity was irrigated as in the previous experiment. A prophylactic dose of 20 cc. was given in the opposite cavity. The infected cavity was irrigated 4 more times during the afternoon; the last aspiration fluid was sterile. On the next day (April 24) 12 cc. of fluid were withdrawn from the left side and 22 cc. from the right side. Both were sterile. On April 26, the rabbit was found dead. Fluid from the left side gave 2,200 colonies per cc.; the right side was heavily infected. The pericardium and the anterior mediastinum contained pus and many streptococci.

Experiments 4 and 5 were very promising, but no better results could be obtained though numerous variations were attempted. When the sodium oleate was omitted, sterilization of the pleural cavity did not take place by the method of irrigating with dilute solutions. Sodium ricinolate was substituted for sodium oleate in two experiments; in three experiments, sodium borate was substituted for the phosphate. In three experiments an unsuccessful attempt was made to treat the uninfected pleural cavity with streptococcus vaccine in the hope that a quickly developing local immunity would prevent the extension of the infection.

This much only was accomplished: the infected cavity had been sterilized, and it had remained sterile for at least 24 hours. Infection of the opposite pleural cavity had been delayed for at least 24 hours beyond the usual time. But there was no way of preventing the escape of the organisms into the pleural lymphatics, the pericardial sac, and the anterior mediastinum. It seems likely that with a less invasive organism, cure of the animal would have been accomplished.

SUMMARY AND CONCLUSIONS

The germicidal titer of acriflavine is markedly influenced by the nature of the medium in which the tests are made. Meat infusion, in particular, lowers the titer.

The action of acriflavine is not altered by the presence or absence of sodium or potassium salts, or of phosphates.

The acriflavine titer is very different at different hydrogen ion concentrations. Alkaline reactions greatly increase the germicidal action.

The adjuvant action of serum is due to its alkaline reaction, which is caused by loss of CO_2.

Certain soaps have an adjuvant action on the acriflavine titer. Of the soaps tested, oleates were found most adjuvant for the streptococcus, laurates for the staphylococcus, and palmitates for the typhoid bacillus.

The metabolic products of the streptococcus here studied antagonize the germicidal action of acriflavine.

The germicidal action of acriflavine is diminished greatly by pus. This is partially the effect of the acidity of the pus, partially the effect of the cells, fibrin, and autolytic products that are present.

A method of treating experimental streptococcus empyema in rabbits was evolved. While this gave some encouraging results, it failed to save any infected animals. This lack of success seemed due to the high virulence of the test organism. The results indicate that acridine dyes, especially when combined with an appropriate soap, would be useful in treating infections that do not tend to become rapidly generalized.

THE EFFECT OF SUPRARENALECTOMY ON ACTIVE ANAPHYLACTIC SHOCK IN THE WHITE RAT

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Kepinow [1] has found that removal of one suprarenal gland and of the greater part of the other from guinea pigs makes them more susceptible to anaphylactic shock, especially in active anaphylaxis. Guinea pigs at best are poor risks for complete suprarenalectomy and Kepinow's use of intravenous injections introduces the additional factor of trauma, to which all suprarenalectomized animals are more susceptible than normal animals. On the other hand, the rat, which withstands comparatively well bilateral suprarenalectomy, has been considered very refractory to anaphylaxis. Parker and Parker,[2] however, showed that it could be produced by injecting intravenously the shocking dose about 11 days after sensitization, explaining Longcope's [3] failure to produce it because the latter used a time interval of more than 22 days. We have in this study determined the effect of bilateral suprarenalectomy on active anaphylactic shock in the white rat, using intraperitoneal injections of horse serum.

METHODS

The rats used in these experiments came from a colony of albino rats of the Wistar stock. In general the control and experimental animals were of about the same age and were kept under similar conditions of feeding and general care.

The horse serum was obtained through the courtesy of the New York City Board of Health, and was frequently tested for natural toxicity on normal and non-sensitized suprarenalectomized rats by intraperitoneal injections. Most of the animals were injected with the same lot of serum, but a few were injected with different lots, and these are identified in the tables. Some of the latter lots were found to lose their natural toxicity by filtration through a Berkefeld filter or by heating to 55 C. for ½ hour. Also some of these lots were preserved with chloroform, but these serums were heated to 55 C. for ½ hour before using, to drive off the chloroform, and like the others were controlled on non-sensitized animals. Sensitizing doses were observed for untoward effects in several of the animals, none of which died from these doses, and no symptoms were observed except in rat 4, which received two sensitizing doses of a toxic serum.

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[1] Compt. rend. Soc. de biol., 1922, 87, p. 327.
[2] J. M. Res., 1924, 44, p. 263.
[3] J. Exper. Med., 1922, 36, p. 627.

All operations were done under ether anesthesia. Both suprarenal glands were removed at one time by the dorsal route.

The rats, after the operation, were kept in individual cages in a warm room for 1 to 2 days, and later the cages were kept covered for additional protection. Otherwise they were given the usual routine care.

All of the controls and some of the suprarenalectomized rats had blood taken for precipitin tests by cutting off the tip of the tail with a sharp scissors. This operation resulted in the death of some of the suprarenalectomized animals, and so was abandoned.

The injection of horse serum intraperitoneally requires only a few seconds and entails a minimum of trauma. After injection, the animals were observed every few minutes for possible symptoms.

TABLE 1

SENSITIZATION AND REINJECTION AFTER SUPRARENALECTOMY

Number	Weight, Gm.	Intraperitoneal Sensitization Days After Suprarenalectomy	Reinjected Days After Last Previous Dose	Intraperitoneal Dose	Result	Remarks
1	240	10, 12, and 14 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm.	Died in 1 hr.	Bled 1 cc. from tail 3, 6, and 9 days after last sensitization
2	200	10, 12, and 14 days (1 cc. per 100 gm.)	11	0.5 cc. per 100 gm.	Died in 3 hrs.	Life prolonged 1½ hrs. (estimated) by giving epinephrine about ¼ hr. before otherwise expected time of death
3	275	9 days (only 1 sensitizing dose) (1 cc. per	11	0.5 cc. per 100 gm.	Moderate symptoms ¾ hr.	
			10	1 cc. per 100 gm.	Moderate symptoms 1¼ hrs.	
			6	1 cc. per 100 gm.	Died in 6½ hrs.	
4	306	10, 12, and 14 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm. (Lot III)	Moderate symptoms 2 hrs.	Died 71 days after operation from traumatic shock. A regenerated suprarenal cortex was found

Complete necropsies were made on all rats and those having chronic pneumonia or in which regenerated suprarenal tissue was found are so identified in the tables.

Four rats were sensitized and reinjected after suprarenalectomy. Six rats for controls were sensitized and reinjected, then their suprarenals were removed; and later they were injected again without further sensitization. Six rats were sensitized, then their suprarenals were removed, and later they were reinjected.

SENSITIZATION AND REINJECTION AFTER SUPRARENALECTOMY

The data for this group are summarized in table 1. Rat 4 was sensitized with a toxic serum producing on the first injection slight abdominal

cramps, slightly prolonged expiration, and a little weakness for one hour; on the 2nd injection 2 days later, there were slight cramps for 20 minutes; and on the 3rd injection 2 days later, after putting the serum through a Berkefeld filter, there were no symptoms. The similarity of these anaphylactoid symptoms to anaphylactic symptoms is well known.

The fact that rat 3 received but one sensitizing dose may have been a factor in its showing milder symptoms.

Rat 4 had relatively mild symptoms. This may be partly explained by the fact that at the time of the operation one suprarenal was incompletely removed, and at necropsy 71 days later there was found wedged between the kidney and the liver a regenerated suprarenal gland about normal size, which microscopically consisted only of cortex.

Rat 4 received, following the first dose after sensitization, 13 injections of horse serum intraperitoneally at varying intervals up to the time of death. As some of these injections were with toxic serums they are not included in the table. The fact that the animal died from the traumatic shock of taking blood at such a late period after suprarenalectomy is in part to be explained by the continued damage of these injections to the resistance of the animal, possibly by damaging the residual suprarenal tissue.

Table 1, compared with table 2, shows how suprarenalectomized animals developed severe to fatal effects from the reinjection of serum, while non-suprarenalectomized animals having control operations developed mild symptoms, or none, from the same procedures. The shocking was done 25 days or more after suprarenalectomy, while the animals were in good condition and not under the influence of the effects of the operative trauma.

CONTROLS: SENSITIZATION AND REINJECTION WITH SUPRARENALS INTACT, THEN SUPRARENALECTOMY AND SUBSEQUENT INJECTION

The control operations consisted of the same procedures as on the suprarenalectomized animals, except that the suprarenals were left intact. None of these operations caused death, and the animals after one day were as active as untreated animals.

Reinjection, after suprarenalectomy, caused moderate to fatal symptoms, while before suprarenalectomy it produced only mild symptoms or none. This increased reaction occurred with only the degree of sensitization that was left after the first shock (table 2).

TABLE 2

CONTROLS. SENSITIZED AND REINJECTED WITH SUPRARENALS INTACT. THEN SUPRARENALECTOMY AND SUBSEQUENT INJECTION

Number	Weight, Gm.	Control Operation	Intraperitoneal Sensitization, Days After Control Operation	Reinjected Days After Last Sensitizing Dose	Intraperitoneal Dose	Result	Suprarenalectomy, Days After Last Injection	Subsequent Injection, Days After Previous Injection	Intraperitoneal Dose	Result	Remarks
5	270	Yes	9, 11, 13 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm.	No symptoms	2	12	1 cc. per 100 gm.	Died in 4½ hrs.	
6	286	Yes	9, 11, 13 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm.	Slight symptoms for 1 hr.	2	12	0.5 cc. per 100 gm	Moderate symptoms 1 hr.	Died 5 days later of suprarenal insufficiency
7	220	Yes	9, 11, 13 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm.	No symptoms	2	12	0.5 cc. per 100 gm.	Moderate symptoms ¾ hr.	Slight chronic pneumonia; gangrene of tail; died 12 hrs. after last dose
								11	1 cc. per 100 gm.	Severe symptoms 2 hrs.	
								12	1 cc. per 100 gm. (Lot II)	Severe symptoms 2 hrs.	
								11	1 cc. per 100 gm. (Lot III)	Severe symptoms 2 hrs.	
8	280	Yes	9, 11, 13 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm.	Slight symptoms 1 hr.	2	—	—	—	Died 10 days after operation
9	336	No	3 doses, 1 every other day (1 cc. per 100 gm.)	14	2 cc. per 100 gm.	No symptoms	1	9	1 cc. per 100 gm.	Severe symptoms 2 hrs.	Killed 74 days after operation
10	291	Yes	12, 14, 16 days (1 cc. per 100 gm.)	11	1 cc. per 100 gm.	Very slight symptoms 25 min.	8	10	0.5 cc. per 100 gm.	Died in 6 hrs.	Life prolonged 3 hr. (estim.) by epinephrin given ¾ hr. before otherwise expected time of death

It is to be noted that rat 9, which had relatively mild symptoms from the second reinjection 9 days after suprarenalectomy, lived 74 days (killed), showing that the degree of prostration from the suprarenal insufficiency was comparatively slight. This may account for the relatively minor effect of the anaphylactic shock. The fact that no gross suprarenal tissue was found at autopsy in this case does not exclude the presence of microscopic accessory cortical tissue.

SENSITIZATION WITH SUPRARENALS INTACT, THEN SUPRARENALECTOMY AND REINJECTION

The animals, except 16, had been subjected to operations before sensitization, but these have no bearing on this experiment, and were done originally with another experiment in mind. On the 3rd and 6th day after sensitization, blood was taken from the tail for precipitin tests. Sensitization was done every other day for 3 days, and 1 cc. of serum per 100 gms. body weight was injected intraperitoneally each time.

Rat 16 was not suprarenalectomized until 64 days after sensitization, yet an injection 2 days later was capable of producing moderate symptoms, showing that a state of slight sensitization persisted (table 3). Two subsequent injections, 1 and 3 days apart for purposes of further sensitization, produced very slight symptoms, and injection 11 days later, or 17 days after suprarenalectomy, produced death as in series 1.

This series shows that the effect of suprarenalectomy in increasing the sensitiveness to anaphylactic shock is quite independent of the process of sensitization, since sensitization was done in this series with the suprarenals intact.

SYMPTOMS OF ANAPHYLACTIC SHOCK

The chief symptom of anaphylactic shock in the suprarenalectomized rat, after the intraperitoneal injection, is that of weakness. The animal moves around less, later stays in one spot, then rests its body on the ground, later its head, then remains in such a position even when stimulated, later lies over on its side, and finally becomes unconscious. Quite characteristic symptoms are the intermittent spasms which are manifested by the animal flattening its belly on the ground and crawling. The respiratory symptoms are also prominent, manifested by prolonged, slower expiration, a quick gasping, deeper inspiration, often ending in a sudden jerk, and a slowing of the respiration often from 100-120 to 60-70 per minute. Scratching is often the first symptom, manifested by the animal at first cleaning itself more frequently, then

attacking local areas persistently, especially the hind quarters, and finally savagely clawing at some spot until exhausted from the effort. Towards the end the animal has a terminal convulsion, it becomes unconscious, the respiration gradually ceases, and the heart continues to beat for about five minutes more. Exophthalmos is observed often at the height of the symptoms. Frequent defecation is often noted, usually early, and sometimes a mucoid diarrhea develops. Cyanosis is observed when the respiratory symptoms are marked. The symptoms

TABLE 3

SENSITIZATION WITH SUPRARENALS INTACT, SUPRARENALECTOMY, REINJECTION

Number	Weight, Gm.	Suprarenalectomy. Dose After Last Sensitizing Dose	Reinjection. Days After Operation	Intraperitoneal Dose	Result	Remarks
11	247	9	6	0.5 cc. per 100 gm.	Moderate symptoms 3 hrs.	Died 4 days after last dose of suprarenal insufficiency
			17	0.5 cc. per 100 gm.	Moderate symptoms 1½ hrs.	
12	292	11	2	1 cc. per 100 gm.	Died in 1¾ hrs.	
13	285	11	2	0.5 cc. per 100 gm.	Died in 4 hrs.	
14	258	9	3	0.5 cc. per 100 gm.	Died in 5¼ hrs.	
15	208	9	3	0.5 cc. per 100 gm.	Died in 1½ hrs.	Chronic pneumonia present
16	340	64	2	1 cc. per 100 gm.	Moderate symptoms 1 hr.	Chronic pneumonia present
			3	1 cc. per 100 gm.	Slight symptoms 15 min.	
			6	1 cc. per 100 gm.	Very slight symptoms 5 min.	
			17	1 cc. per 100 gm.	Died in 2¼ hrs.	

start in 5 to 20 minutes after injection, usually about 15 minutes, and increase in intensity up to ½ to 2 hours after injection, then either disappear gradually or persist till death. The respiratory symptoms and scratching usually occur first, then the spasms and weakness develop. The spasms gradually disappear, and finally the respiratory symptoms and weakness either persist till death or gradually disappear.

The relatively late onset and the long duration of the symptoms before death may be due to the slow absorption from the peritoneum compared with the intravenous route, and also the toxic action of the

secondarily produced anaphylactoxins, which as shown by Manwaring and his coworkers [4] in the case of the dog are formed chiefly in the liver.

The symptoms and pathology are similar to those recorded by Parker [2] by intravenous injections into normal rats, if allowances are made for the less rapid absorption.

ANATOMICAL CHANGES

Congestion and hemorrhage were noted in the 11 rats dying of anaphylactic shock as follows: In all, marked congestion and petechial hemorrhages were observed in the gastro-intestinal tract, especially in the stomach, small intestine, and cecum, and slight in the colon, with bloody mucus in the small intestine in 9; in 8, congestion of the lungs, one of which had hemorrhages; in 9, marked general peripheral vascular dilatation, including the peritoneum; in 7, congestion of the liver; in 5, congestion of the lymph glands, 3 of which had petechial hemorrhages; in 4, congestion of the spleen and brain; in 2, congestion of the tubes, ovaries, and thymus.

Additional gross changes in these 11 rats dying of anaphylactic shock, and attributed to the effects of suprarenalectomy, are as follows: in 9, apparent enlargement of the lymph glands; in 8, apparent enlargement of the thymus; in 4, granular degeneration of the kidneys; in 8, diminution of body fat. In 3 cases there was chronic pneumonia.

Anaphylactic shock and suprarenalectomy have these effects in common: diarrhea, congestion and hemorrhages, but to a much less extent after suprarenalectomy than after acute anaphylactic shock.

RELATION OF PRECIPITINS TO ANAPHYLAXIS

Parker [2] and Longcope [3] found that the precipitins in the white rat were usually present in the blood 4 to 10 days after sensitization, and Parker found that the most severe shock occurred when no precipitins and very little antigen were present in the serum.

In the controls precipitin and precipitinogen tests were done 3, 6, and in some cases 9 days after the last sensitizing dose. In 7 cases, precipitin tests were done at 3 days after the last sensitizing dose, 2 giving titers of 1-100 dilution of horse serum, 3 of 1-50, and 2 of 1-10. At 6 days, in 10 cases, 1 gave 1-100, 3 of 1-50, 4 of 1-10, and 2 were negative at 1-10. At 9 days, in 3 cases, 1 gave 1-50, 1 of 1-10, and 1 was negative at 1-10. Rat 16 had a titer of 1-50 from 3-31 days, then 1-10 till 48 days, and negative on 52 and 56

[4] Proc. Soc. Exper. Biol. & Med., 1922, 20, pp. 182, 184.

days after operation. Moderate shock symptoms were produced in this rat 66 days after sensitization, and 16 days or so after the precipitin test became negative.

In 6 cases precipitinogen tests for residual horse serum in the rat blood were done 3 days after the last sensitizing dose, 1 giving a titer of 1-10,000, 1 of 1-5,000, 3 of 1-1,000, and 1 of 1-500. At 6 days, in 10 cases, 2 gave titers of 1-1,000, 4 of 1-500, and 4 of 1-100. At 9 days, 2 cases gave titers of 1-100. In rat 16, in 10 tests, from 10 to 56 days after sensitization, no horse serum was demonstrable.

In general these findings agree with the findings of precipitin formation of other workers as Spain and Grove,[5] Parker,[2] and Longcope.[3]

GENERAL OBSERVATIONS

Thirty-five rats were suprarenalectomized; 11 died from anaphylactic shock, 18 from suprarenal insufficiency (natural causes plus absence of suprarenals), 5 from shock of taking blood, and 1 was killed. Of the 18 dying from suprarenal insufficiency, 7 died in the first 2 days, and 6 of these had hemorrhage in the abdomen; 3 died in 3 to 5 days, one having abdominal hemorrhage and one extensive chronic pneumonia; and 8 died 10 to 34 days after suprarenalectomy.

In some of the earlier operations hemorrhage, traumatic shock, and pneumothorax plus the suprarenal insufficiency produced death in 1 to 2 days while controls similarly traumatized but having suprarenals left intact did not show any effects from the operation. This shows how double suprarenalectomy lowers the resistance to the effects of traumatic shock in 1 to 2 days after operation.

SUMMARY

Of a group of 4 white rats sensitized 9 to 14 days after bilateral suprarenalectomy and given intraperitoneal injections of horse serum 11 days later, 2 died and 2 had moderate symptoms. Of a group of 5 rats sensitized 9 to 11 days before suprarenalectomy and given injections 2 to 6 days after the operation, 4 died and 1 had moderate symptoms. Of a group of 6 control rats similarly sensitized and given a later dose 3 had no symptoms and 3 had slight symptoms. These results indicate that bilaterally suprarenalectomized rats show a markedly increased susceptibility to anaphylactic shock, produced by intraperitoneal injections of horse serum. This effect is obtained whether the animals are sensitized before or after suprarenalectomy, and seems related chiefly to the degree of suprarenal insufficiency.

[5] J. Immunol., 1925, 10, p. 433.

UNSUCCESSFUL EXPERIMENTS WITH MERCUROCHROME AS A BILIARY ANTISEPTIC

IX. EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS

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For a number of years one of us (K. F. M.) has studied intermittingly the effect of a large series of metallic inorganic and organic compounds, dyes, etc., on rabbits infected with B. typhosus in the gall-bladder or in one of the kidneys. Since the negative results differ in no way from those of Uhlenhuth and Messerschmidt,[1] Hailer and his associates,[2] Beckwith [3] and others, a report of the extended tests is deemed unnecessary. Occasionally an experimental series would develop promising "leads" which, however, were promptly contradicted by subsequent tests. Among the preparations which were tried out, mercury compounds took a prominent part. Although the cystin mercury preparation, $(SCH_2\text{-}CHNH_2\text{-}COO)_2$ Hg of Stuber [4] was not available, colloidal mercury, mercurophen and calomel were thoroughly investigated. The last named substance was chosen on account of the definite cures which were reported by Haibe [5] on a series of 64 convalescent human carriers. According to this worker an alternating treatment with calomel and turpentine oil for a period of four weeks gave the most favorable results. In experiments on rabbits this procedure, extended over 6 weeks, failed to cure the gall-bladder carrier state. Similarly negative results were noted with other mercury compounds irrespective of the vigorous treatment which produced in a number of animals definite symptoms of mercurialism. In this connection it is well to emphasize the relative low tolerance of rabbits for preparations of mercury, a fact also recorded by Uhlenhuth and Messerschmidt, Young, Hill and White [6] and others. Mercurophen a highly potent antiseptic

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[1] Deutsch. med. Wchnschr., 1912, 51, p. 2397; 1920, 46, p. 1293.
[2] Arb. a. d. k. Gsndhtsamte., 1914, 47, pp. 303, 451, 470; 1914, 48, p. 80.
[3] J. Infect. Dis., 1921, 29, p. 495; 1923, 33, p. 457.
[4] München. med. Wchnschr., 1918, 65, p. 790.
[5] Mém. cour. Acad. roy. d. med. d. Belge, Bruxelles, 1921, 22, p. 1.
[6] Arch. Surg., 1925, 10, p. 813.

for B. typhosus in the test-tube (Schamberg, Kolmer and Raiziss [7] failed in the tolerated dose of 0.001 to 0.0015 gm. per kg. of body weight to sterilize the biliary passages but produced in several animals a definite hemorrhagic nephritis. The numerous enthusiastic reports [6] on the value of dibrom-oxymercury fluorescein or mercurochrome-220 soluble, as a general antiseptic in infections and infectious diseases lead some time ago to a series of experiments with this particular substance. At first the general reactions were followed on a few animals. The results were by no means encouraging and the work originally contemplated was discontinued. However, renewed interest in mercurochrome as a chemotherapeutic agent for the sterilization of the typhoid carrier was created by the paper of Hill and Scott.[8] These workers concluded from a number of in vitro and in vivo tests that the compound possesses excellent properties as a biliary antiseptic. In fact they reported that mercurochrome in vitro is actively bactericidal in a bile medium, killing B. typhosus (Rawlings strain) when exposed for one hour in a dilution of 1 : 1000 or for 24 hours in a dilution of 1 : 50,000. Furthermore their data indicated that it was possible to sterilize the bile of four rabbits infected with B. typhosus in the gall-bladder. This result was accomplished with intravenous injections (1 to 7 doses of 3 to 5 mg. per kilo) and with treatment for one week with two administrations daily of the drug by the stomach tube (7.5 mg.). As far as the literature indicates and the experiences of this laboratory amply confirm, this is the first report in which the chemotherapeutic treatment of the typhoid carrier state in the rabbit has yielded 100% cures in the treated, and an equal percentage of infection in the untreated. Obviously a repetition of this work appeared most desirable. Since the brilliant results of Hill and Scott have not been duplicated a series of in vitro tests which may in part explain the failures have been carried out. It is the purpose of this communication to describe briefly these experiments.

IN VIVO TESTS WITH MERCUROCHROME

Two series of rabbits infected with B. typhosus have been treated with mercurochrome. The first is an incomplete preliminary experiment and is merely cited in order to indicate the failure of the drug to sterilize other foci than the gall-bladder.

Series 1.—Five rabbits were immunized with four injections of a standard typhoid vaccine. Twenty-nine days after the last injection each animal received

[7] J. Infect. Dis., 1919, 24, p. 551.
[8] Arch. Int. Med., 1925, 35, p. 503.

intravenously approximately 1.5 billion living B. typhosus, grown on blood agar slants, per kilo of weight. Two recently isolated strains, no. 19 and no. 21, were used. The usual general reactions were definite and in accordance with the observations detailed in former papers. Three rabbits of the series were treated intravenously on the 4th and 8th day after the infection with 10 to 15 mg. of mercurochrome. At necropsy two of the three were found to harbor typhoid bacilli in the bile and in the kidneys. The third presented at necropsy no macroscopic lesions and the cultures of the various organs remained sterile. Of the two controls one showed typical carrier lesions and the bile gave an abundant growth of B. typhosus, while the other appeared anatomically and culturally normal. The findings on one rabbit are of special interest, the abbreviated history is therefore given below.

Rabbit 2603: Weight, 2850 gm., has been immunized by four injections of typhoid vaccine; on the 29th day after the last injection of the vaccine 4.5 billion B. typhosus are given intravenously; the animal is very toxic, refuses its food. Weight on 8th day 2125 gm., intravenous injection of 7.5 mg. mercurochrome on the morning of the 7th, 7.5 mg. in the afternoon of the 8th and 5.0 mg. on the 11th day. The animal remains toxic and continues to lose weight. Killed on 22nd day. Weight 1900 gm.

Necropsy: Cadaver of an emaciated male rabbit; some fluid in the abdominal cavity; gall-bladder distended, whitish, thick walled and injected; bile light greenish, with yellowish, coarse granular sediment; liver slightly mottled; both kidneys enlarged, pale grayish edemia of renal pelvis; yellow-grayish streaks in myocardium; bone marrow soft grayish. Cultures on brilliant green plates revealed B. typhosus from the following organs: bile, gall-bladder wall, liver, urine, kidneys, duodenum and faeces, enrichment in peptic broth gave also positive cultures of the bone marrow.

This experimental series is incomplete and too small. However, in comparison with similar series in which other drugs or dyes were tested, certain general conclusions appear justifiable. Mercurochrome is less efficacious on B. typhosus in vivo than for example methyl-violet, fuchsin, new fast green or its analogue new solid green (Hoffman-La Roche, Basel) (1 and 3). Since the presence of typhoid bacilli in the gall-bladders of the injected animals was not controlled by cystic punctures it is not unlikely that two of the rabbits had either no biliary infections or spontaneously recovered from the localization of the bacteria. In any event the absence of a significant sterilizing effect of mercurochrome in a rabbit with bone marrow foci and the presence of typhoid bacilli in the kidneys of two treated animals suggested that the preparation is not only ineffective but harmful. Localization and persistence of B. typhosus in the kidneys of rabbits following intravenous inoculations is rare [9] and one could not evade the impression that the administration of a mercury preparation might have served as a predisposing factor. Subsequent tests have, however, shown that conditions other than the mercurochrome must have been responsible for the prolonged localiza-

[9] Meyer, Neilson and Feusier: J. Infect. Dis., 1921, 28, p. 456.

TABLE 1

TREATMENT OF GALL-BLADDER CARRIERS WITH MERCUROCHROME-220 SOLUBLE RABBITS IMMUNIZED AND SUBSEQUENTLY INFECTED BY INTRACYSTIC INOCULATIONS

Treatment				Necropsy		
Number	Day After Infection	Method	Total Amount of Mercurochrome	Killed, Days After Last Treatment	Macroscopic Lesions	Positive Cultures From
1	18 and 20th	2 intravenous injections	29 mg	14	Typical gall-bladder lesions, colorless bile with sediment	Bile and gall-bladder wall
2	18 and 20th	2 intravenous injections	32.5 mg	16	Typical gall-bladder lesions, colorless bile with sediment	Bile and liver
3	18 and 20th	2 intravenous injections	33.0 mg.	16	Typical gall-bladder lesions, colorless bile with sediment	Bile, mesenteric lymph nodes
4	18 and 20th	2 intravenous injections	35.0 mg.	16	Typical gall-bladder lesions, colorless bile with sediment	Bile and gall-bladder wall
5	18th	1 intravenous injection	13.0 mg.	22	Typical gall-bladder lesions, colorless bile with sediment	Bile, liver, gall-bladder, intestines
6	18, 20, 22, 24, 26, 28, 30th	7 intravenous injections	120.4 mg	6	Typical gall-bladder lesions, colorless bile with sediment	Bile, gall-bladder, duodenum
7	18, 20, 22, 24, 26, 28, 30th	7 intravenous injections	129.5 mg.	6	Typical gall-bladder lesions, colorless bile with sediment	Bile and gall-bladder
8	18, 19, 20, 21, 22, 23rd	2 daily feedings for 6 days	291.6 mg.	6	Typical gall-bladder lesions, colorless bile with sediment	Bile and gall-bladder
9	18, 19, 20, 21, 22, 23rd	2 daily feedings for 6 days	300 mg.	6	Typical gall-bladder lesions, colorless bile with sediment	Bile, gall-bladder, duodenum
10	18, 19, 20, 21, 22, 23rd	2 daily feedings for 6 days	300 mg	6	Gall-bladder small whitish, bile dark	Negative
11	Control.....			18	Typical gall-bladder lesions, colorless bile with sediment	Bile, liver and gall-bladder
12	Control.....			34	Typical gall-bladder lesions, colorless bile with sediment	Bile and bone-marrow
13	Control.....			36	Typical gall-bladder lesions, colorless bile with sediment	Bile, gall-bladder, duodenum
14	Control.....			42	Typical gall-bladder lesions, colorless bile with sediment	Bile, bone-marrow, mesenteric lymph nodes, intestines
15	Control.....			30	Gall-bladder wall thick; green bile with sediment	Negative
16	Control.....			42	Typical gall-bladder lesions, colorless bile	Bile, duodenum and mesenteric lymph nodes

tion of B. typhosus in the kidneys of the rabbits studied in series 1. Influenced by the observations just reported further experiments were discontinued until the paper of Hill and Scott [8] had appeared. A second carefully controlled series was immediately planned and executed. Since it is well known [10] that intravenous inoculations produce persistent gall-bladder infections in only 65% of the injected animals, direct intracystic inoculations, a procedure developed by Uhlenhuth and Messerschmidt, were used in the preparation of the carrier state.

Series 2.—Sixteen rabbits varying in weight from 2900 to 3400 gm. were immunized with a standard typhoid vaccine; one subcutaneous, two intraperitoneal and one intravenous injection of 1 billion organisms were given. Nine days after the last injection each animal was laparotomized under ether anesthesia; the gall-bladder was punctured, a small amount of bile was aspirated and 0.1 cc. of a 24 hour old broth culture of B. typhosus was inoculated. A series of silk sutures closed the peritoneal cavity. Fecal examinations with the aid of brilliant green bile enrichment repeatedly demonstrated the elimination of B. typhosus. On the 18th day after the intracystic inoculation, 10 rabbits were treated by various routes and with various doses of mercurochrome. Six animals served as controls. The protocols of the treatment and the necropsy findings are recorded in table 1.

The results given need little comment, they are conclusive. Nine of the 10 gall-bladder carriers remained infected irrespective of the intensive treatment with mercurochrome. One rabbit which had received 300 mg. of mercurochrome by stomach tube gave sterile cultures although the gall-bladder exhibited the residual changes of an inflammatory process. An untreated animal presented the same lesions. Previous studies [9] conducted in this laboratory support the observations of Hailer and his associates so far that 15 to 20% of the rabbits infected by intracystic inoculations of B. typhosus spontaneously recover in the course of from two to three weeks. It must be borne in mind by every experimenter that spontaneous recovery of the carrier state in rabbits is the rule rather than the exception. In the light of these facts it is impossible to ascribe the apparent cure in rabbit 10 to the treatment with mercurochrome. There are two noteworthy differences between the treated and untreated animals. Two of the control rabbits gave positive bone marrow cultures, while the same tissues of the treated animals remained sterile. Furthermore the typhoid bacillus was present in the left liver lobes of two rabbits injected with small doses (13.0 and 32.5 mg.) of mercurochrome and absent in the organ of the more intensively treated animals. By merely considering the animals of this series one might be temped to look upon these observations as indicative of the germicidal value of mercurochrome for B. typhosus in the tissues

[10] Christiansen, Neilson and Meyer: J. Infect. Dis., 1921, 28, p. 404.

other than the gall-bladder. However, experiences extending over 10 years have shown, that similar variations are quite common in untreated animals and the only conclusion one is really justified to draw from experimental series 2 is that mercurochrome is ineffective in the treatment of the experimental typhoid carrier state of rabbits.

Since the work of Hill and Scott could not be confirmed the question immediately arose, "Are the typhoid strains used in the experiment mercury tolerant or are physiological-pharmacological factors responsible for the failures"? In order to shed light on these problems a number of in vitro and in vivo tests have been carried out.

IN VITRO AND IN VIVO TESTS WITH RABBIT BILE

In the experiments to be recorded the following phases have been investigated:

(1) The germicidal properties of mercurochrome dissolved in hepatic rabbit bile on B. typhosus no. 19 and no. 21.

(2) The excretion and the concentration of mercurochrome in hepatic bile after an intravenous injection of the compound.

(3) The germicidal action of the hepatic bile of rabbits treated with mercurochrome.

In order to secure comparative data the three determinations were conducted with the secretions of the same rabbit. Hepatic bile was aseptically collected for several hours through a temporary common duct fistula. The technic described in a previous article has been employed.[11] It is important to emphasize that hepatic bile alone was used in the experiments. Hill and Scott [8] collected hepatic bile through a drainage tube inserted in the fundus of the gall-bladder, although they repeatedly speak of gall-bladder bile. The physiological studies conducted by workers of this laboratory on rabbits, guinea-pigs, cats and dogs and subsequently enhanced by Rous and McMaster [12] have demonstrated that retention and concentration in the gall-bladder is necessary to transform the hepatic secretion into cystic bile. Several comparative studies have demonstrated that such a condition never exists when a drainage tube is placed in the fundus of the gall-bladder. For bacteriologic work collection by common duct fistula has many advantages since contaminations are rare and the flow is remarkably regular, a feature which is particularly valuable in elimination studies. At first the bile has been collected under paraffine oil in order to preserve the P_H of the secretion.

[11] Neilson and Meyer: J. Infect. Dis., 1921, 28, p. 511.
[12] J. Exper. Med., 1921, 34, p. 47.

This precaution was later discarded since a difference in the P_H from 7.6 to 8.4 did not influence materially the germicidal properties of the mercurochrome present in the bile.

After a few preliminary experiments had been carried out the following general technic was used for the in vitro tests:

0.9 cc. of bile were placed in small tubes. Dilutions were made by adding 0.1 cc. of mercurochrome dissolved in bile or distilled water, ten times the final concentration of the drug, to 0.9 cc. of bile. Each dilution was seeded with two loopful of an 18 hour old broth culture of B. typhosus no. 19 and no. 21 mixed, and carefully shaken. Immediately after the inoculation and at regular intervals thereafter one loopful of the mixture was plated and transferred to peptic digest broth. The necessary controls to test the bactericidal properties of the bile specimen, etc., were included in the series. The samples of bile collected before but mixed with mercurochrome and those secured after the intravenous inoculation of the drug were tested simultaneously under identical conditions and incubated at 37 C.

TABLE 2

THE GERMICIDAL PROPERTIES OF MERCUROCHROME IN THE BILE OF RABBIT 2

In Vitro							In Vivo *								P_H 8.4
	Growth, Hours after Inoculation						Hours after Injection Bile Collected	Mercurochrome Estimated Amount	Growth, Hours after Inoculation						
Dilutions Made	Immediately	½	1	2	4	24			Immediately	½	1	2	4	22	Cc. of Bile in Each Sample
1:1,000	+	0	0	0	0	0									
1:5,000	+	+	0	0	0	0									
1:7,000	+	+	+	0	0	0	½	1:6,000	+	+	+	+	+	0	4.2
1:8,000	+	+	+	+	0	0									
1:10,000	+	+	+	+	0	0	1	1:10,000	+	+	+	+	+	0	5.0
							2	1:15,000	+	+	+	+	+	0	6.2
							3	1:17,000	+	+	+	+	+	0	7.0
							4	1:20,000	+	+	+	+	+	+	6.1
							5	1:21,000	+	+	+	+	+	+	5.5
							6	1:20 000	+	+	+	+	+	+	5.0
							7	1:22,000	+	+	+	+	+	+	4.3
1:25,000	+	+	+	+	+	0									
1:50,000	+	+	+	+	+	+									
Total															43.3
Average flow per hour															6.2

* 22 mg. of mercurochrome injected intravenously (5 mg. per kg.). 3.04 mg. or 13.8% of mercurochrome recovered in the bile.

The comparative in vitro and in vivo-in vitro test carried out on four rabbits furnished the following interesting information:

Rabbit 1: Weight 4200 gm. Bile was collected for five hours before 21 mg. of mercurochrome were administered intravenously. The in vitro tests showed that in hepatic bile (P_H 8.4), and mercurochrome in a concentration of 1:5,000 killed B. typhosus in one hour and a dilution of 1:50,000 in 24 hours. The bile specimens collected for a period of six hours after the injection of the drug containing mercurochrome in the concentration of 1:7,300 to 1:30,000 failed to kill the test organisms.

Rabbit 2: Weight 4300 gm. Bile was collected for four hours before 22 mg. of mercurochrome were injected intravenously. The results of the in vitro and the in vivo-in vitro tests are summarized in table 2.

Rabbit 3: Weight 3300 gm. Bile was collected for five hours before 17 mg. of mercurochrome were injected intravenously. The drug dissolved in hepatic bile killed B. typhosus in a concentration of 1 : 5,000 in 30 minutes, 1 : 10,000 in 6 and 1 : 25,000 in 24 hours. The bile specimens collected for two hours beginning immediately after the injection of mercurochrome destroyed B. typhosus in vitro in 24 hours. The estimated concentration of mercurochrome was 1 : 6,000 in the first, 1 : 7,000 in the second and 1 : 10,000 in the third specimen.

Rabbit 4: Weight 2400 gm. Bile collected for four hours before 18 mg. of mercurochrome were inoculated intravenously. The results of the in vitro and the in vivo-in vitro tests are presented in table 3.

TABLE 3

THE GERMICIDAL PROPERTIES OF MERCUROCHROME IN THE BILE OF RABBIT 4

In Vitro						In Vivo *							Pa 8.4
	Growth, Hours after Inoculation							Growth, Hours after Inoculation					
Dilutions Made	Immediately	2	4	6	24	Hours after Injection Bile Collected	Estimated Amount	Immediately	2	4	6	24	Cc. of Bile in Each Sample
1:5,000	+	+	0	0	0								
1:7,000	+	+	0	0	0								
1:8,000	+	+	0	0	0	1	1:8,000	+	+	+	0	0	11.8
1:10,000	+	+	0	0	0								
						2	1:20,000	+	+	+	+	0	13.0
1:25,000	+	+	0	0	0								
						3	1:35,000	+	+	+	+	+	12.5
						4	1:45,000	+	+	+	+	+	13.7
1:50,000	+	+	+	+	+	5	1:50,000	+	+	+	+	+	12.0
						6	1:60,000	+	+	+	+	+	9.4
						7	1:70,000	+	+	+	+	+	7.7
1:75,000	+	+	+	+	+								
						8	1:90,000	+	+	+	+	+	8.5
1:100,000	+	+	+	+	+	9	1:100,000	+	+	+	+	+	8.8
Total													97.4
Average flow per hour													10.8

* 18 mg. of mercurochrome injected intravenously (7.5 mg. per kg.). 3.48 mg. or 19.3% of mercurochrome recovered in the bile.

The above experiments establish the following significant facts:

(1) Mercurochrome dissolved in hepatic bile of rabbits is germicidal; a dilution of 1 : 5,000 destroys at least 10,000,000 typhoid bacilli inside of from ½ to 4 hours. Greater dilutions, as for example, 1 : 25,000, require at least 24 hours to kill the same number of bacteria.

(2) Bile specimens of rabbits which had been injected intravenously with 5 mg. of mercurochrome per kilo of weight may exhibit definite bactericidal properties and destroy 10,000,000 typhoid bacilli in 24 hours. The excretion collected during the first two, and occasionally the first three hours are antiseptic. An injection of 7.5 mg. of mercurochrome per kilo body weight renders the bile secreted during the first hour slightly more germicidal than that of rabbits treated with 5 mg.; the sterilization is accomplished in six hours. Some rabbits inoculated with mercurochrome in the tolerated amount excrete a bile which is not

bactericidal irrespective of the fact that the coloring of the specimens suggests the presence of the drug. An attempt was made to determine the actual amount of mercurochrome excreted with the hepatic bile. Previous experience in connection with other tests had shown that for example congo-red or typhoid bacilli injected intravenously would appear in the fistular bile in from 4 to 18 minutes after the inoculation. Furthermore, it was frequently noted that the excretion of the dye preceded the appearance of corpuscular elements (bacteria) in the bile from 8 to 15 minutes. The coloring of the bile specimens left no doubt that a color compound passed rapidly from the blood stream through the liver into the bile. However, it was of importance to determine (1) the actual amount secreted and (2) to demonstrate, if in any way possible, the presence of mercury in the bile. In analyzing the data it appeared not unlikely that the mercurochrome is partially combined or decomposed in its passage through the liver or the antiseptic is modified through interaction with bile substances and the blood proteins. It is naturally possible that a part of the metal is retained in the organ while the dye is excreted. One of us (H. S.) has therefore attempted a quantitative estimation of mercurochrome in the bile specimens which have been studied bacteriologically.

SECRETION OF MERCUROCHROME IN BILE

The four different methods which have been tried are briefly described:

"Compensator" Method.—The procedure described by Rosenthal[13] has been followed. A sample of the excreted colored bile in a test-tube is placed in the front of a tube containing distilled water. This tube is compared in a suitable comparator box as used for P_H determinations with a specimen of bile collected before the administration of mercurochrome which stands in front of an aqueous standard dilution of the drug.

"Schlesinger Solution" Method.—The bile pigments are precipitated by Schlesinger solution (10% solution of zinc acetate in alcohol) and the clear samples are compared with standard mercurochrome dilutions. Hill and Scott found this procedure quite satisfactory.

"Nitrite" Method.—The bile pigments are discolored by the addition of nitrous acid; alkali is then added and the samples compared with standard solutions.

"Comparison" Method.—The bile specimens are compared with dilutions of mercurochrome prepared in bile of the same animal.

The relative value of these methods is clearly demonstrated in tables 4 to 7.

The "Compensator" method yields estimations which are unquestionably too high. According to tables 4, 5 and 6, 18.5, 69 and 125% of the injected amount of mercurochrome were recovered in the fistula

[13] Proc. Soc. Exper. Biol. & Med., 1923, 21, p. 2271.

bile during an observation period of from 6 to 7 hours. These evidently impossible results are not due to the inexactness of the readings since independent determinations 24 hours apart (table 6) furnished nearly identical results.

In explaining these observations it is reasonable to assume that mercurochrome and a substance of the bile, probably one of the pigments,

TABLE 4

Determination of Mercurochrome in Bile

Experiment 1. 21 Mg. Mercurochrome-220 Injected

Collection, Hours after Injection	Cc. of Bile	Concentration of Mercurochrome-220 in Bile: 1. "Compensator" Method		2. "Schlesinger Solution" Method	
		Dilution	Mg.	Dilution	Mg.
½	7.2	1:7,300	0.99	1:7,500	0.96
½ 1	8.9	1:7,500 9,000	1.08	1:11,000	0.81
2	9.5	1:15,000	0.63	1:17,500	0.54
3	7.6	1:15,000 20,000	0.43	1:20,000	0.38
4	7.1	1:20,000	0.36	1:40,000	0.18
5	5.5	1:22,000	0.25	1:60,000	0.09
6	4.2	1:30,000	0.14	1:100,000	0.04
Total 6	50.0 = 8.3 per hour		3.88 mg. = 18.5% of total amount injected		3.00 mg. = 14.3% of total amount injected

TABLE 5

Experiment 2. 22 Mg. Mercurochrome-220 Injected

Collection, Hours after Injection	Cc. of Bile	Concentration of Mercurochrome-220 in Bile: 1. "Compensator" Method		2. "Nitrite" Method	
		Dilution	Mg	Dilution	Mg.
½	4.2	1:1,200	3.5	1:150	28
½ 1	5.0	1:2,000	2.5		
2	6.2	1:3,000	2.1		
3	7.0	1:3,500	2.0		
4	6.1	1:4,000	1.5	1:1,000	
5	5.5	1:4,200	1.3	1:1,000	
6	5.0	1:4,000	1.3		
7	4.3	1:4,500	0.9	1:2,500	
Total 7	43.3 = 6.2 per hour		15.1 mg. = 68.6% of total amount injected		28 mg. = 127% of total amount injected

combine with each other. The resulting new complex is of a deeper color than that anticipated by the mere summation of the colors of the single constituents used in the comparator test. The rate of the reaction depends probably on the composition of the bile and varies from animal to animal. For example in experiment 1 no color complex was formed and the readings corresponded closely with those of the second method.

The disadvantages of the "Compensator" method are partially corrected by the Schlesinger solution. Strongly colored compounds of mercurochrome and bile pigments are immediately dissolved but several other errors render the readings uncertain. If for example Schlesinger reagent is added to a 1 : 5,000 solution of mercurochrome in water, the

TABLE 6

EXPERIMENT 3. 17 MG. MERCUROCHROME-220 INJECTED

Collection, Hours after Injection	Cc. of Bile	Concentration of Mercurochrome-220 in Bile: 1. "Compensator" Method, Dilution	Mg.	24 Hours Later	2. "Nitrite" Method, Dilution	Mg.	3. "Comparison" Method, Dilution	Mg.
½ }	4.8	1:1,000	4.80	.....	1:170	28.2	1:6,000	0.80
½ } 1	5.7	1:1,300	4.38	1:1,200	.....	....	1:7,000	0.81
2	10.5	1:2,300	4.57	1:2,400	.....	....	1:10,000	1.05
3	8.5	1:3,500	2.43	1:3,000	.....	....	1:15,000	0.57
4	7.8	1:3,900	2.00	1:3,500	.....	....	1:15,000	0.52
5	4.4	1:4,000	1.10	1:4,000	.....	....	1:20,000	0.22
6	5.3	1:4,000	1.32	1:4,000	.....	....	1:20,000	0.26
½	2.8	1:4,000	0.70	1:4,500	.....	....	1:20,000	0.14
Total 6½	49.8 = 7.7 per hour		21.30 = 125% of total amount injected			28.2 = 166% of total amount injected		4.37 = 25.7% of total amount injected

TABLE 7

EXPERIMENT 4. 18 MG. MERCUROCHROME-220 INJECTED

Collection, Hours after Injection	Cc. of Bile	Concentration of Mercurochrome-220 in Bile "Comparison" Method, Dilution	Mg.
1	11.8	1:8,000	1.48
2	13.0	1:20,000	0.65
3	12.5	1:35,000	0.36
4	13.7	1:45,000	0.30
5	12.0	1:50,000	0.24
6	9.4	1:60,000	0.16
7	7.7	1:70,000	0.11
8	8.5	1:90,000	0.09
9	8.8	1:100,000	0.09
Total 9	97.4 = 10.8 per hour		3.48 mg. = 19.3% of total amount injected

fluorescence rapidly disappears and the solution is of a stronger reddish tinge than the standard. After an elapse of several minutes depending somewhat on the concentration of the zinc in the reagent some of the mercurochrome precipitates; the remaining dye solution is then weaker in color although of the usual mercurochrome tinge. In the presence of bile the precipitation is apparently more complete than in the aqueous

standard comparison tube. Thus the method is apt to yield as a rule readings which are too low. In experiment 1 (table 4) a negative error of approximately 23% as compared with the compensator method was noted. According to the laws of adsorption the error increases with the degree of dilution. Hench, Snell and Greene [14] also realized the disadvantage of the Schlesinger solution and discolored the bile by the addition of nitrous acid. The data presented in tables 5 and 6 indicate, however, that this procedure furnishes readings which are far too high. In experiment 2, 127%, and in experiment 3, 166% of the total amount of mercurochrome injected appeared in the fistular bile during the first 30 minutes of collection. On the other hand the nitrous acid procedure of removing bile pigments furnishes additional evidence which supports the contention that mercurochrome enters into a reaction with some of the bile substances. Simple test-tube experiments have repeatedly shown that mercurochrome and bile treated separately with nitrous acid fail to yield the deep red color which ordinarily develops when a mixture of the drug and the secretion is subjected to the action of the same reagent.

The finally adopted "Comparison" method gave readings which are in close agreement with each other. However, it is not unlikely that this method has also some inherent defects since the combining power of the bile for the dye may vary in the course of the experiment. In fact, it may be low at the beginning of the collection and then increase under the influence of the mercurochrome injection. Or the complex which is formed while the drug passes through the liver into the bile may be different from that which is developed when bile and mercurochrome are mixed in the test-tube.

The data of the four experiments definitely indicate that a part of the intravenously injected drug is excreted in the bile. Concerning the actual amounts one is left without dependable information, in fact the bacteriological tests suggest that the amounts colorimetrically established are still too high or that the bactericidal properties of the excreted mercurochrome are changed. In this connection the data of experiments 2 and 3 are again recalled; mercurochrome dissolved in hepatic bile destroyed B. typhosus in a dilution of 1 : 10,000 inside of four hours, while the colored fistula bile containing colorimetrically mercurochrome in a dilution of 1 : 6,000 required at least 22-24 hours to kill the same bacteria under identical conditions. Samples of fistular bile containing the excreted dye have in every experiment been less germicidal than the corresponding mercurochrome-hepatic bile solutions.

[14] Jour. Pharmacol. and Exper. Therap., 1925, 25, p. 142.

Since the differences cannot be attributed to changes in H-ion concentration of the bile in the course of the tests it was thought advisable to estimate the mercury content in the excreted bile. The small amount of material available did not lend itself to quantitative determinations. In fact it is quite certain that in several tests the mercury escaped detection entirely. The sublimation method was used for all the analyses.

The tissues or body fluids were decomposed with Conc. HCl and a $KClO_3$ solution on a moderately boiling water-bath. The clear fluid was filtered and the remaining fat several times extracted with the same chlorine-solution. An excess of $KClO_3$ was destroyed and the solution adjusted to a slightly acid reaction. The mercury was then precipitated on the surface of granular zinc, the metal thoroughly washed with water, alcohol and ether. The zinc was finally heated in a test-tube the upper part of which was drawn out to a capillary. By examining the area of condensation under the microscope and by adding some iodine crystals, the mercury was readily identified.

The mercury droplets were examined under the microscope or with the aid of a hand-lens and the amount roughly estimated by comparing the deposit with standard samples prepared in an identical manner. Mercury was found in the fistular bile of rabbits 2 and 3, but could not be demonstrated in the secretion of rabbit 4. The actual amount in the bile of rabbit 2 was estimated at 0.5-1.0 mg. This value corresponds within the limits of error to the amount of mercurochrome (3.0 mg.) which was estimated colorimetrically. In experiment 4 several organs of the rabbit were examined for their mercury content. Positive tests indicated the presence of the metal in the liver, the lungs, the kidneys, and the intestines, urine and heart blood, while negative findings were recorded for the gall-bladder wall, spleen and bone-marrow.

The demonstration of mercury in two of three specimens of fistular bile removes every doubt that mercurochrome given intravenously is not excreted through the liver. Furthermore, the yield of the metal corresponds fairly closely to the amount of mercurochrome determined by the colorimetric comparison method. It is obviously permissible to conclude that the figures in experiments 1 to 4, tables 2 and 3, represent the actual amounts of the drug excreted in the bile. Thus in rabbit 1 there was an average biliary excretion of 18.5% in 6 hours, rabbit 3, 25.7% in 6½ hours, and in rabbit 4, 19.3% in 9 hours after injection. These data are at variance with those of Rosenthal and White,[15] who estimated by the "Compensator" method the average biliary excretion of mercurochrome as 25 per cent in one hour and 44% in 2 hours after injection.

[15] Jour. Pharmacol. and Exper. Therap., 1924, 24, p. 265.

The fallacies of the "Compensator" method have already been discussed and it has been pointed out that the readings secured by this procedure are frequently too high.

The contention of Hill and Rosenthal that by intravenous administration mercurochrome reaches the bile quickly and persists in strong concentrations for at least 2 to 4 hours is supported by the facts detailed in this paper. However, the deductions that under these conditions a strong germicidal action must be expected is contradicted by the in vitro tests. Irrespective of the high concentration of the drug in the fistular bile, the destruction of the typhoid bacilli is slow and usually requires an exposure of 24 hours. One cannot evade the conclusion that mercurochrome in its passage through the liver becomes modified and loses a part of its antiseptic properties. One could speculate concerning the various factors which might be responsible for this modification in healthy rabbits. Extensive physiological tests combined with in vitro studies would be necessary to secure dependable data concerning the influence of the diet, the hepatic function, the biliary flow and the composition on the germicidal properties of the bile of normal rabbits intravenously inoculated with mercurochrome. Such a comprehensive investigation was not in the scope of this inquiry. Moreover, these experiments would be of little value without a consideration of the physiology of the biliary excretion and the gall-bladder in the course of a typhoidal infection. In this connection the following questions suggest themselves: (1) Is mercurochrome actually discharged into the gall-bladder? (2) Does the hydrogen ion concentration, and (3) the inflammatory exudate increase or decrease the germicidal properties of the drug dissolved in the gall-bladder bile? These questions will be briefly discussed. Hill and Scott assumed from their fistular experiments that mercurochrome is regularly excreted into the gall-bladder. Direct tests were not carried out. In fact very little work has been done on this subject.[16] Auster and Crohn [17] studied the appearance of phenoltetrachlorphthalein in hepatic bile and in gall-bladder bile. They observed on dogs that the dye in the bile is discharged into the duodenum, and in the gall-bladder bile at about the same time, 10 to 15 minutes after administration. The manner in which the gall-bladder is filled in the rabbit, the time during which bile enters it, and other factors with regard to the passage of bile into it are unknown. Only a small fraction (2%) of the daily biliary excretion enters into the gall-bladder [11] and

[16] Mann: Physiol. Rev., 1924, 4, p. 251.
[17] Am. J. M. Sc., 1922, 164, p. 345.

it is reasonable to assume that the intravenously injected mercurochrome may never reach this organ.

In order to secure comparative data the following experiments were carried out:

Rabbit 1, (weight 3400 gm.), on a liberal oat and hay diet, received intravenously 20 mg. of mercurochrome, and was killed with strychnine two hours after the injection; 0.75 cc. of limpid bile (P_H 8.0) and 5 cc. urine (P_H 6.6) faintly tinged with the dye were removed. In in vitro tests the bile destroyed 10,000,000 typhoid bacilli in 6 and the urine in 24 hours.

Rabbit 2, (weight 4400 gm), received 25 mg. of mercurochrome intravenously, and was killed 3½ hours later; 1.2 cc. of limpid bile (P_H 8.4) and 20 cc. of deeply fluorescent urine were collected. The bile destroyed the inoculated typhoid bacilli in 4 hours, while the urine still continued viable bacteria after an incubation period of 24 hours.

Rabbit 3, (weight 4000 gm.), received water but no food for 12 hours. The animal received 20 mg. of mercurochrome and was sacrificed 6 hours after the injection. 1.25 cc. of dark greenish, slimy bile (P_H 6.8) and 10 cc. of urine (P_H 6.4) were collected. In both excretions B. typhosus still grew after an incubation period of 24 hours.

The results of the three experiments indicate that mercurochrome may or may not reach the gall-bladder in a concentration which is sufficient to destroy approximately 10,000,000 typhoid bacilli inside of 24 hours. Judging from the consistency the bile present in the gall-bladder of rabbits 1 and 2 was in all probability hepatic bile which had entered the viscus shortly before the animal was killed. In rabbit 2 the bile was slightly more concentrated which may in part have been responsible for the greater germicidal property of the excretion. However, it is barely possible that the decreased hydrogen ion concentration acted in a similar manner. The experiment on rabbit 3 was undertaken with the view of securing a greater concentration of the drug and a correspondingly greater antiseptic action. Just the opposite result was recorded. The cystic bile was neither bacteriostatic nor germicidal; the potent concentrations of the drug were apparently discharged directly into the duodenum. Nothing passed out of the gall-bladder and consequently little or no hepatic bile entered the viscus. Stasis is a common occurrence in the typhoid gall-bladder carrier state of the rabbit in which the cystic duct is frequently blocked by exudate, cellular débris and carbonate sediments. Neither the normal nor the inflamed gall-bladder wall excretes mercurochrome although there is some evidence that the drug may through some unknown movement of the bile pass into the gall-bladder. It reaches, however, the viscus in dilutions which are not antiseptic for the typhoid bacillus.

Even should the mercurochrome enter the diseased gall-bladder in effective concentrations, various other factors may render it impotent. It is generally believed that the substance retains its antiseptic properties in the presence of proteins. A number of observations made in this laboratory fail to support this contention. For example mercurochrome dissolved in rabbit serum fails to destroy typhoid bacilli in a concentration of 1:5,000, while the same dilution in distilled water killed the bacteria under identical conditions in from 4 to 6 hours. Another experiment is even more convincing.

The pleural exudate produced in a rabbit by aleuronate injections was mixed with mercurochrome; the dilutions were seeded with B. typhosus and the progress of sterilization was followed. The data are shown in table 8.

The data presented in table 8 leave no doubt that mercurochrome loses the major part of its antiseptic properties through the presence of proteins, particularly serum or inflammatory exudate. These observations are fully in harmony with the recently published experiments of Hirschfelder, Malmgren and Creavy,[18] who demonstrated the penetration of mercurochrome into the edema fluid in paraphenylendiamin edema in concentrations of from 1 : 5,000 to 1 : 8,000. Irrespective of the high concentration the edema fluid was neither bactericidal nor bacteriostatic for the colon bacillus or the staphylococcus. These observations lend support to the belief that the reduced germicidal property of fistular bile of rabbits inoculated with mercurochrome is due to the addition of serum or other protein fluids which accompany the excretion.

The reaction of the bile may either increase or decrease the properties of mercurochrome. Cystic bile of infected gall-bladders has as a rule a hydrogen ion concentration of P_H 7.4-7.5, which on standing

TABLE 8

Action of Mercurochrome in the Presence of an Inflammatory Exudate in Vitro

Dilutions	Growth at Intervals after Inoculation					
	Immediately	15 Min.	1 Hour	4 Hours	6 Hours	24 Hours
In exudate 1:2,000	+	+	+	+	+	+
1:5,000	+	+	+	+	+	+
1:10,000	+	+	+	+	+	+
In distilled water 1:5,000	+	+	+	+	0	0

increases to P_H 8.4-9.0. On the other hand the concentrated bile of normal gall-bladders is usually slightly acid (P_H 6.4 to 6.6.). Young, White and Swartz[19] in their first publication and several independent tests conducted in this laboratory attest to the greater germicidal property of mercurochrome in an alkaline medium. In fact the drug is frequently twice as potent in bile with a P_H of 8,6 than in the same medium with a P_H of 6.6. This behavior is probably of some advantage in the treatment of experimental gall-bladder carriers in which the secretion is usually slightly alkaline.[11] Unfortunately the favorable reaction is neutralized by the serum and the cellular protein material present in the chronically inflammed gall-bladder.

In conclusion it is recalled that the in vitro, and the in vivo and in vitro tests, explain fully the negative results obtained in the treatment of experimental typhoid gall-bladder infections of the rabbit. However, throughout the discussions it was assumed that the bile alone is infected.

[18] Jour. Pharmacol. and Exper. Therap., 1925, 24, p. 459.
[19] J. Am. M. A., 1919, 73, p. 1483.

Little consideration was given to the established fact that the gall-bladder wall harbors nests of bacteria from which the bile is constantly reinfected. The treatment of these foci was not investigated since it has been pointed out in a former paper [9] that chronic carriers, particularly rabbits, infected by direct inoculations of the gall-bladder, develop lesions which cannot be treated pharmaceutically and usually present a therapeutic difficulty which is rarely encountered in human cases. Appreciating these limitations, it is obvious that the negative therapeutic results obtained in rabbits should not be unreservedly applied in drawing analogies as to the possibility of treating similar conditions existing in man. Furthermore, it must be realized that in human cholecystitis the infection is found most commonly in the wall, while the bile is sterile (Alvarez, Meyer, Rusk, Taylor and Easton).[20] The causative organism is usually a Streptococcus or a representative of the coccus group. A chemotherapeutic preparation suitable for these infections must not only be a biliary antiseptic but must also penetrate in effective concentrations the gall-bladder wall and must be germicidal for cocci. According to Walker and Sweeney [21] the latter requirement is only partially fulfilled by mercurochrome and again the experimental studies reported here and elsewhere lend little support to the assumption that the drug is a potent tissue antiseptic.

However, this does not preclude the possibility as Churchman [22] in his admirable discussion has recently pointed out, that mercurochrome which under experimental conditions is ineffective, may prove its worth in clinical use. In any case if clinical trials of mercurochrome are made it should be kept in mind that oral administration of the drug is less dangerous than intravenous injections, and again effective concentrations over prolonged periods may be readily secured with less inconvenience to the patient than repeated intravenous medication.

CONCLUSIONS

Mercurochrome intravenously injected is excreted in the hepatic bile of rabbits in concentrations which may destroy 10,000,000 typhoid bacilli in from 6 to 24 hours. However, it has been impossible to cure experimentally produced gall-bladder carriers by giving mercurochrome intravenously or by mouth.

The colorimetric methods used in the estimation of mercurochrome, the influence of proteins, etc., on the germicidal properties of the compound are discussed.

[20] J. Am. M. A., 1923, 81, p. 974.
[21] Jour. Pharmacol. and Exper. Therap., 1926, 26, p. 466.
[22] J. Am. M. A., 1925, 85, p. 1849.

A BACTERIOLOGIC STUDY OF CHRONIC PERIAPICAL DENTAL INFECTION

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Accurate knowledge of the bacteriologic status of the pulpless tooth is important in studying the relation of dental infection to systemic disease. The clinician is especially interested in determining the accuracy and dependability of the diagnostic methods commonly employed in detecting periapical infection.

Miller[1] in his classic studies on dental bacteriology gave scant attention to chronic periapical infection. Moody[2] cultured fifty-five chronic alveolar infections and found the streptococcus viridans as the predominant organism. Hartzell and Henrici[3] cultured 282 cases of chronic alveolar infections and found streptococci either pure or contaminated with staphylococcus albus. Lucas[4] made 181 cultures of which 155 were positive. A variety of organisms were obtained, the streptococcus being most predominant. Smith[5] in dental infections in children, found streptococci of all types, pneumococci, and staphylococci. Rickert[6] using dextrose brain broth, made cultures from the apex of over 200 pulpless teeth and found 51% positive and 49% negative. The cultures were made from curettings of the exposed apex before extraction. The observers quoted have employed only liquid culture mediums.

To obtain further data concerning this subject I have cultured the apex and periapical tissues from 1,500 teeth. Deep tubes of glucose brain broth agar and glucose brain broth, which afford all gradations of oxygen tension, have been used as culture mediums. The P_H is approximately 7.0. The nutritive qualities are especially favorable for the growth of the organisms in infections about the teeth. The agar medium shows also the number of bacteria in the material inoculated, which can be grown. Only sufficient agar is added to barely solidify. This does not retard growth yet holds the colonies discrete. The broth medium is used for the inoculation of animals, for transplants to blood agar plates, and for identification of the organisms present by the gram stain. The method of preparation has been described elsewhere.[7]

In obtaining the material for culture the field of operation was prepared by first scrubbing well with gauze, the teeth to be extracted. The teeth and gums were then painted with iodine, the iodine was removed with alcohol and the area of operation walled off with sterile gauze. The tooth was extracted with sterile forceps. After extraction the apex of the tooth was cut off with a sterile instrument directly into a tube containing about 1 cc. of sterile 0.85% sodium chloride and a small amount of sharp sand. In making the culture the tube containing

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[1] Die Microorganismen der Mundhohle, 1892.
[2] J. Infect. Dis., 1916, 19, p. 515.
[3] J. Am. Dent. A., 1915, 2, p. 333.
[4] Dental Cosmos, 1920, 62, p. 1197.
[5] Arch. Pediatrics, 1919, 36, p. 148.
[6] J. Am. Dent. A., 1922, 9, p. 300.
[7] Haden, R. L.: Arch. Int. Med., 1923, 32, p. 828.

the root tip was well shaken to macerate the tissue on the tip of the tooth. The culture material was poured into a deep tube of glucose brain agar which had been heated and cooled to 40 C. The tube was quickly inverted once and allowed to harden. The small amount of salt solution remaining in the tube was poured into a tube of glucose brain broth. The inoculated tubes are incubated at 38 C. for from 24 to 48 hours. Usually the maximum growth takes place in 24 hours.

Gram stains were routinely made of the positive broth cultures.

For determination of type. transplants were made from the broth tubes to blood agar plates. Single colonies of streptococci were picked from the agar plates and transferred to a tube of brain broth, and incubated. From this tube transplants were made to the carbohydrate broth tubes.

CLINICAL MATERIAL

Apical cultures have been made from over 3,000 teeth. For statistical purposes I have included here 1,500 cultures from incissors, cuspids, and bicuspids only which had been extracted without contamination. Controls are necessary to determine the chances of error. The best controls are obviously cultures of vital teeth employing the same technic as in culturing the pulpless teeth. I have constantly cultured vital teeth for this check, thus affording a base line for the interpretation of results obtained in pulpless teeth. The material was obtained from patients entering a pay dental clinic. They were largely ambulant patients who came primarily for dental treatment. Some were under medical treatment in a hospital for systemtic disease. Others, while ambulant, were referred by physicians for the removal of dental foci which were considered related etiologically to systemic disease.

The radiographs have been made by a uniform method of exposure and development giving constant results. At least 14 films were taken for each patient, affording at least two views of each tooth.

I have divided the teeth cultured into three groups: vital, pulpless and negative in the radiograph, and pulpless and positive in the radiograph. I have classified as vital those teeth which respond to the electric current. In these the pulp functions though not necessarily normally. Teeth which show no response to stimuli have been classified as pulpless. These are certainly pulpless from the standpoint of function, regardless of whether the pulp is still in position though dead, or has been mechanically removed. There is no common agreement as to exactly what teeth should be placed in the radiographic group. I have taken a conservative attitude.

QUANTITATIVE RESULTS OF CULTURES

Four hundred cultures were made from vital teeth (table 1). It is assumed that vital teeth without caries are sterile, hence positive cultures in this group are taken as indicating only the chances of technical error; 85.5% showed no growth in the agar tube, and 45% were sterile in the broth culture. The high percentage of positive broth cultures from vital teeth emphasizes the futility of using broth only as a routine culture medium. It is apparent that positive cultures in broth from pulpless teeth mean little when such a high percentage of positive cultures are obtained from vital teeth. Of the 14.5% positive cultures in agar, 9.8% contained less than 10 colonies per tube. Only 1.2% of the total contained as many as 100 colonies per tube. 4.8% of the 400 cultures showed 10 or more colonies per tube.

The results of cultures of pulpless teeth are of value only in comparison with the result of cultures of vital teeth. As the best basis for comparison I have arbitrarily selected the groups of positive cultures in which the agar tube contained 10 or more colonies. The results of the cultures from vital teeth indicate such contaminating organisms as were found were usually less than ten per tube.

Five hundred radiographic positive, pulpless teeth were cultured (table 1); 26.6% showed no colonies in the agar shake tube, 44.2% have over 100 colonies and 73.4% have one or more colonies; 62.8% had 10 or more colonies. A high percentage of the positive agar cultures showed a large number of organisms, 9% of this group were sterile in broth.

TABLE 1

RESULTS, IN %, OF APICAL CULTURES FROM 1,500 TEETH

		Deep Agar Tube					Broth	
			Positive					
Group	Total Number	Negative	0–10 Colonies	10–50 Colonies	50–100 Colonies	Over 100 Colonies	Positive	Negative
Vital	400	85.5	9.8	2.8	0.8	1.2	55.0	45.0
Pulpless teeth with negative radiograph	600	44.3	9.5	16.7	4.0	25.7	83.8	16.8
Pulpless teeth with positive radiograph	500	26.6	10.6	11.4	4.2	44.2	91.0	9.0
All pulpless	1,100	36.3	10.0	14.3	4.0	34.1	87.1	12.9

Six hundred pulpless teeth with negative radiographs have been cultured (table 1); 44.3% of these showed no organisms in the agar shake tube; 25.7% had over 100 colonies and 56.7% 1 or more colonies. The comparative group containing 10 or more colonies per tube, constitute 46.2% of the total number. 16.8% were sterile in broth.

The most noteworthy finding in the roentgen-ray negative group is the high percentage showing bacteriologic evidence of infection without radiographic evidence. The incidence of infection is nearly as high as in the radiographic positive group. Considering all pulpless teeth together, 36.3% showed no colonies in the agar tube, 34.1% had over 100 colonies, and 64.6%, 1 or more colonies, 53.7% had 10 or more colonies, 12.9% were sterile in broth.

The cultures included in this report have been made over a period of three years. The percentages have varied little. In the first 254 cultures from roentgen-ray positive teeth, 60.6% showed 10 or more colonies, while for the total of 500 the percentage is 62.8.

TYPES OF ORGANISMS

A smear was routinely made from the broth culture and stained by gram. In the 1,091 positive broth cultures a gram negative bacillus was found once. In 963 cultures, only streptococci were found. Streptococci were mixed with staphylococci in 9 cultures, and with gram positive bacilli in 37. Streptococci were present, alone or mixed with other organisms, in 1,009 or 92.5% of the 1,091 positive cultures. Gram positive bacilli alone were found 58 times, staphylococci only 19 times, and the two together 3 times.

TABLE 2

ORGANISMS FOUND IN APICAL CULTURES AS IDENTIFIED BY GRAM STAIN OF BROTH CULTURE

Organism	Number from Vital	Number from Pulpless, Radiographic Negative	Number from Pulpless, Radiographic Positive	Total Number
Streptococcus only	157	416	390	963
Staphylococcus only	13	4	2	19
Staphylococcus and streptococcus	3	5	1	9
Gram positive bacillus only	23	28	7	58
Gram positive bacillus and streptococcus	4	12	21	27
Gram positive bacillus and staphylococcus	1	2	0	3
Gram negative bacillus only	0	0	1	1
Total	201	467	423	1,091

Some of the stains of streptococci grew in long chains, but the most common type was a diplococcus. The diplococcus is usually elongate, often lanceolate, and thus closely resembles the pneumococcus. The morphologic appearance, however, varies greatly with the culture medium. An organism appearing in diplococcus form in broth may show very long chains in agar.

The types of organisms found in the various groups are shown in table 2. The staphylococci and gram positive bacilli occur much more frequently in the vital group than in the pulpless teeth.

Of the positive broth cultures, 346 were transferred to blood agar plates. 302 were found to be pure cultures, usually of non-hemolytic streptococci. Only three times were hemolytic streptococci found; twice in pure culture and once associated with a staphylococcus aureus. There were 44 mixed cultures. In the 66 cultures from roentgen-ray positive teeth, only 3 were found mixed on blood agar plates. The non-hemolytic streptococci were usually green on blood agar. Not infrequently, however, the colonies were grey.

For the identification of the non-hemolytic streptococci by fermentation reactions single colonies were picked from the blood agar plates to broth tubes. From the broth, after incubation, transplants were made to the various sugar tubes. The results of the determination of type (Holman classification) of 305 strains of non-hemolytic streptococci are shown in table 3. The fecalis, mitis, and salivarius are the common strains. Streptococcus salivarius occurs much more frequently in the cultures from vital teeth than from the pulpless teeth.

TABLE 3

DISTRIBUTION OF STREPTOCOCCI FROM APICAL BROTH CULTURES ACCORDING TO HOLMAN CLASSIFICATION

Organism	Number from			Total	
	Vital	Pulpless			
		Radiographic			
		Negative	Positive	Number	%
Streptococcus fecalis	19	64	62	145	47.5
Streptococcus mitis	14	24	25	63	20.7
Streptococcus salivarius	15	14	15	44	14.4
Streptococcus non-hemolyticus I	5	16	8	29	9.6
Streptococcus non-hemolyticus II	3	6	4	13	4.3
Streptococcus non-hemolyticus III	0	3	1	4	1.3
Streptococcus ignavus	0	4	3	7	2.3
Total	56	131	118	305	100.0

SUMMARY

The results of periapical cultures from 1,500 incisor, cuspid and bicuspid teeth are reported.

All cultures have been made in deep tubes of glucose brain broth and glucose brain agar which affords most favorable nutritive conditions, varying oxygen tension and allows the number of bacteria to be estimated.

Vital teeth have been routinely cultured to determine the chances of error in technic.

The quantitative results of cultures from different types of teeth are given.

For comparative purposes the number of tubes containing 10 or more colonies is most valuable. 4.8% of the vital group, 62.8% of the pulpless teeth with positive radiograph and 46.2% of the pulpless teeth with negative radiograph showed 10 or more colonies per tube.

The non-hemolytic streptococcus is by far the most commonly found organism. Hemolytic streptococci only rarely occur.

The most common types of streptococci as determined by carbohydrate fermentation are the fecalis, mitis, and salivarius.

MUCOUS MEMBRANE IMMUNITY TO PNEUMOCOCCI IN RABBITS

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Hope for the future in the prevention of pneumococcus pneumonia probably depends on the production of a suitable antigen, and definite knowledge concerning the best method for its administration. Much work with this idea in view already has been done.

That pneumococci could be recovered from the deepest parts of the lungs of mice as long as 18 hours after they had been sprayed with a pneumococcus culture was demonstrated by Wherry and Butterfield.[1] It was also noticed that although a virulent culture of pneumococcus was used, none of 29 mice which were permitted to live following the inhalations became infected.

Stillman[2] obtained similar results and in addition chilled his mice before spraying, at the time of spraying, and after spraying but without any noticeable effect on increasing the susceptibility of the mice to infection. He also attempted to lower the local resistance of the mice by inducing injuries to the mucous membrane by allowing the mice to inhale ether before or after spraying, but these attempts also failed to increase the frequency of infection.

Wadsworth[3] found that one injection of 3 billion pneumococci, type 1, afforded only slight protection to mice against type 1, but that three injections of the vaccine afforded definite protection against a 0.0001 cc. fatal dose and finally that 6 weekly doses of 6 billion cocci, type 1 protected against a virulent inoculation of 0.001 cc. but not against 0.01 cc. of the virulent type 1 culture. "The degree of protection that was obtained was not great considering the quantities of vaccine that were used to vaccinate the animals."

Perlzweig and Steffen[4] noticed that a moderate immunity was induced in mice by one subcutaneous injection of a pneumococcus type 1 vaccine, and that a much higher degree of immunity was obtained when two injections were given.

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[1] J. Infect. Dis., 1920, 27, p. 315.
[2] J. Exper. Med., 1923, 38, p. 117.
[3] J. Infect. Dis., 1920, 27, p. 315.
[4] J. Exper. Med., 1923, 38, p. 163.

Stillman and Branch [5] found that nonimmunized mice which had been alcoholized and had died of septicemia following exposure to a spray of living pneumococci rarely showed any localization of infection in the lungs. However mice which had been partially immunized to pneumococci by previous inhalations of living or dead pneumococci often developed pneumococcus lobar pneumonia if they were exposed to a spray of virulent pneumococci while alcoholized.

Stillman [6] found that a definite degree of active immunity could be induced in mice through the repeated inhalation of live pneumococci while only slight immunity was induced by the repeated inhalation of killed pneumococci.

Cecil and Blake [7] working with monkeys found that subcutaneous inoculation with pneumococcus type 1 vaccine in doses comparable to those employed in man did not protect monkeys against subsequent attacks of pneumococcus type 1 pneumonia. Vaccination did however modify the course of the disease and invasion of the blood stream by the pneumococci was usually slight or absent in vaccinated animals.

Cecil and Steffen [8] later found that the subcutaneous inoculation of monkeys with three large doses of pneumococcus type 1 vaccine conferred on them a complete immunity against experimental pneumococcus type 1 pneumonia. They also found that the intravenous inoculation of small doses of pneumococcus type 1 vaccine completely protected the monkeys from the homologous type.

Cecil and Steffen [9] were able to completely immunize monkeys against experimental pneumococcus type 1 pneumonia by intratracheal inoculation with three doses of pneumococcus type 1 vaccine given at 5 to 7 day intervals. Spraying of the throat did not produce complete immunity. The immunity established by the intratracheal injection of pneumococcus vaccine resulted in little or no demonstrable protective substances in the serum of these monkeys.

Later Cecil and Steffen [10] were successful in immunizing monkeys against pneumococcus type 2 pneumonia by giving them three subcutaneous injections of pneumococcus type 2 vaccine. They were similarly successful in vaccinating against pneumococcus type 4. However the subcutaneous injection of monkeys with three doses of pneumococcus type 3 vaccine conferred immunity in only 50% of the animals.

[5] Ibid., 1924, 40, p. 733.
[6] Ibid., p. 567.
[7] Ibid., 1920, 31, p. 519.
[8] Ibid., 1921, 34, p. 245.
[9] U. S. Pub. Health Rep., Reprint No. 796, 1922, p. 2735.
[10] J. Exper. Med., 1923, 38, p. 149.

Mackenzie[11] working with guinea-pigs found that intraperitoneal injections of killed and living pneumococci type 1 resulted in a high degree of active immunity and a serum with strong protective power.

Wadsworth[12] succeeded in immunizing rabbits against intravenous inoculation of virulent broth cultures by giving them 6 intravenous injections of 1 cc. each of pneumococcus type 1 vaccine. Also rabbits which were given 12 intravenous injections of a heated broth culture were protected against subsequent inoculation with living broth cultures.

Bengston[13] found marked protection given rabbits which had received 3 intravenous injections of 1 cc. of pneumococcus type 1 vaccine. The test infecting doses of live culture were given intraperitoneally. It was also observed that this immunity in rabbits against type 1 pneumococcus lasted to a moderate extent for 6 months and had disappeared in 9 months.

Davidson[14] found that 6 intravenous injections of a heated saline suspension of pneumococcus type 1, or of a heat killed broth culture protected rabbits against fatal infecting doses of virulent pneumococci, type 1.

PRELIMINARY STUDIES ON METHODS OF IMMUNIZATION

A highly virulent culture of pneumococcus type 1 was used. The vaccine for immunization was composed of a 24 hour blood agar slant culture of this organism suspended in 10 cc. of physiological salt solution, thoroughly washed, resuspended, and sterilized by heating at 70 degrees C. for one hour. The resultant vaccine had a fairly heavy turbidity.

The susceptibility of rabbits to pneumococci when introduced through an abrasion in the buccal mucous membrane was determined by scratching the buccal surface of one cheek and rubbing in one small loopful of a 24 hour blood agar slant culture. Three rabbits so treated died within 12 hours of marked pneumococcus septicemia.

Five rabbits were then injected daily for 10 days with 1 minim of the vaccine introduced just beneath the buccal mucous membrane of the right cheek. Ten days after the last vaccination these rabbits were tested for immunity by rubbing one loopful of a 24 hour blood agar slant culture into a scratch in the vaccinated area. Four of these rabbits survived showing no reaction and remained normal (table 1). One died in about 24 hours. On autopsy no pneumococci could be recovered from the heart blood but there was an extensive pericarditis which was

[11] Ibid., 1925, 41, p. 53.
[12] J. Immunol., 1920, 5, p. 429.
[13] U. S. Pub. Health Service, Hygienic Lab. Bull. No. 139, 1924.
[14] Edinburgh M. J., 1925, 32, p. 67.

apparently of several days' duration. Experience has shown us that non-immune rabbits inoculated in this way through a scratch in the buccal mucosa die within 12 to 24 hours of pneumococcus septicemia. Five control rabbits injected at the same time, in the same way, and with the same culture, all died within 24 hours of pneumococcus septicemia.

Three rabbits were vaccinated for 10 consecutive days by introducing 1 minim of the same vaccine subcutaneously on the ventral surface of the abdomen. Ten

TABLE 1

Preliminary Study of Vaccination by Different Routes

Number of Rabbits	Nature of Vaccine	Site of Daily Injection of Vaccine	Number of Doses of Vaccine	Site of Infecting Injection	Results
11	Heated NaCl suspension	5, Submucosa, right cheek	10	Scratch in mucosa, right cheek	4 lived, 1 died*
		1, Subcutaneous, on abdomen	10	Scratch in mucosa, right cheek	Died
		1, Subcutaneous, on abdomen	10	Scratch in mucosa, right cheek	Died
		1, Subcutaneous, on abdomen	10	1 minim, subcutaneous, on abdomen	Died
		1, Intradermal, on abdomen	10	Scratch in mucosa, right cheek	Died
		1, Intradermal, on abdomen	10	Scratch in mucosa, right cheek	Died
		1, Intradermal, on abdomen	10	1 minim, intradermal, on abdomen	Died
9	48 hour autolysate	5, Swab nose and mouth daily	10	Scratch in mucosa of right cheek	All 5 died
		1, Submucosa, right cheek	10	Scratch in mucosa, right cheek	Lived and survived a second injection 17 days later
		3, 1 drop in right eye	10	Scratch in conjunctiva, right eye	1 lived, 1 died after 11 days, 1 died after 12 days
Controls					
10	.....	.....	..	Submucosa, right cheek	All died in 24 hours
2	.....	.....	..	Subcutaneous, on abdomen	
2	.....	.....	..	Intradermal, on abdomen	
2	.....	.....	..	Scratch in conjunctiva	

* Pneumococci were recovered from all the dead rabbits excepting this one.

days after the last vaccination one of these rabbits was injected in the vaccinated area with 1 minim of a suspension of this highly virulent culture and it died in 2 days. The remaining 2 rabbits were given the test infecting dose by rubbing a loopful of a 24 hour blood agar slant culture into a scratch in the mucous membrane of the right cheek. Both of these animals died about the same time as the controls and pneumococci were recovered from the heart blood. This experiment showed that no immunity was derived from the subcutaneous vaccination.

Three rabbits were given the same vaccine intradermally on the ventral surface of the abdomen. Ten days after the last vaccination one of these rabbits was given 1 minim of the infecting dose intradermally in the vaccinated area. It died about the same time as the controls. The other two rabbits were given the infecting dose in a scratch in the buccal mucous membrane of the right cheek and both died. Control rabbits which were injected intradermally with the same culture died within 24 hours.

In another experiment an autolysed broth culture of the same pneumococcus type 1 was used as antigen. One rabbit received 1 minim of this antigen submucously in the right cheek daily for 10 days. Ten days later it withstood a fatal infecting dose introduced in the vaccinated area through a scratch in the buccal mucous membrane. Twenty-seven days after the vaccination it withstood a second fatal infecting dose. Two controls died within 24 hours.

Three rabbits received one drop of this autolysate in the right eye daily for 10 days. Ten days later 2 loopsful from a 24 hour blood agar slant culture were rubbed into a scratch made in the conjunctiva of the vaccinated eye. One of these rabbits survived. The other two died, one after 11 days, and the other after 12 days; pneumococci were not recovered from the heart blood nor had there been any evidence of a local infection in the eye. Two control rabbits were given the same infecting dose from the same culture in a scratch in the conjunctiva of the right eye and both died within 24 hours.

The nostrils and mouths of 5 rabbits were swabbed daily for 10 days with this autolysate. Ten days later they were given the infecting dose in a scratch in the buccal mucous membrane of the right cheek. All died about the same time as the controls of pneumococcus septicemia; the controls infected through a scratch in the buccal mucosa of the right cheek died within 24 hours.

The results of these preliminary studies revealed that the mucous membrane route for vaccination was the only method among those tried which protected rabbits from fatal infecting doses. It is noteworthy that these rabbits were immunized by 10 minims of vaccine, given in one minim doses, 24 hours apart.

FURTHER EXPERIMENTS ON IMMUNIZATION BY THE MUCOUS MEMBRANE

Fifteen rabbits were immunized by introducing 1 minim of the vaccine daily for 10 days into the buccal mucous membrane of the right cheek. The vaccine used in this case was prepared from a pneumococcus type 1, Williams culture, and was prepared in the same way as formerly. At the time of testing the immunity of these rabbits a pneumococcus type 1, Rockefeller Institute culture, was used because the pneumococcus type 1 Williams culture had lost its virulence and it was impossible to build it up again by repeated animal passage. Reference to table 2 shows the results of the attempt to immunize this group of rabbits. Thirteen of the fifteen immunized rabbits withstood the fatal infecting dose of pneumococci when introduced into a scratch in the mucous membrane of the right cheek. or the left cheek, or when injected

with 2 minims of a heavy suspension of the same culture either intravenously, subcutaneously, or intraperitoneally. The two immunized rabbits which died were found to have quite a marked pneumococcus septicemia. Nine control rabbits were given the fatal infecting dose in the same manner as were the immunized rabbits and all died within 12 hours with marked pneumococcus septicemia. It is considered noteworthy that none of the fatalities occurred among those rabbits injected subcutaneously, intravenously, or intraperitoneally.

This experiment brought out two facts. First that it is possible to develop a generalized immunity in the rabbit to the pneumococcus type 1 vaccinating through the buccal mucous membrane, and second that the

TABLE 2

Animals Immunized with Pneumococcus Type 1, Williams, and Tested with Infecting Culture Pneumococcus, Type 1, Rockefeller

Number of Rabbits	Nature of Vaccine	Site of Daily Injection of 1 Minim of Vaccine	Number of Doses of Vaccine	Site of Infecting Injection	Results
15	Heated NaCl suspension	Submucosa, right cheek	10	5, Submucosa, right cheek	4 lived, 1 died, pneumococcus found
				5, Submucosa, left cheek	4 lived, 1 died, pneumococcus found
				2, Intraperitoneal	2 lived
				2, Subcutaneous	2 lived
				1, Intravenous	1 lived
Controls					
2	.....	.....	..	Submucosa, right cheek	All died in 12 hours
2	.....	.....	..	Submucosa, left cheek	All died in 12 hours
2	.....	.....	..	Intraperitoneal	All died in 12 hours
2	.....	.....	..	Subcutaneous	All died in 12 hours
1	.....	.....	..	Intravenous	All died in 12 hours

immunity developed against the pneumococcus type 1, Williams, protected against a heterologous strain of pneumococcus type 1, Rockefeller.

The second experiment was very similar to the first with the exception that the vaccine was prepared from the pneumococcus type 1, Rockefeller culture. The results are found in table 3 where it is seen that 10 rabbits survived the fatal infecting dose. Of those which did not survive there were no signs in 3 of a pneumococcus septicemia. Nine control rabbits injected in the same manner as were the immunized animals all died within 12 hours with marked pneumococcus septicemia.

The results of this experiment substantiate the previous work in showing that it was possible to stimulate the production of a generalized

TABLE 3

ANIMALS IMMUNIZED WITH PNEUMOCOCCUS, TYPE 1, ROCKEFELLER

Number of Rabbits	Nature of Vaccine	Site of Daily Injection of 1 Minim of Vaccine	Number of Doses of Vaccine	Site of Infecting Injection	Results
15	Heated NaCl suspension	Submucosa, right cheek	10	5, Submucosa, right cheek	5 lived
				5, Submucosa, left cheek	2 lived 1 died 3rd day, pneumococci found 1 died 5th day, no pneumococci 1 died 6th day, no pneumococci
				2, Intraperitoneal	2 lived
				2, Subcutaneous	1 lived 1 died 7th day, no pneumococci
				1, Intravenous	1 died 5th day, pneumococci found
Controls					
2	.....	....	..	Submucosa, right cheek	All died in 12 hours
2	.....	.....	..	Submucosa, left cheek	
2	.....	.....	..	Intraperitoneal	
2	.....	.....	..	Subcutaneous	
1	.....	.....	..	Intravenous	

TABLE 4

TESTS FOR IMMUNITY FOUR MONTHS AFTER IMMUNIZATION

Number of Rabbits	Nature of Original Vaccine	Site of Daily Injection of 1 Minim of Vaccine	Site of Infecting Injection	Results
8	Pneumococcus 1, Rockefeller	Submucosa, right cheek	5, Submucosa, right cheek	4 lived 1 died, no pneumococci found
			2, Submucosa, left cheek	2 died, pneumococci found
			1, Intraperitoneal	Died, pneumococci found
11	Pneumococcus 1, Williams	Submucosa, right cheek	4, Submucosa, right cheek	3 lived 1 died, poor, pneumococci found
			3, Submucosa, left cheek	2 lived 1 died, pneumococci found
			2, Intraperitoneal	2 lived
			1, Subcutaneous	1 lived
			1, Intravenous	1 lived
Controls				
4	....	.....	Submucosa, right cheek	All died in 12-24 hours
4	.....	.....	Submucosa, left cheek	
2	.....	.....	Subcutaneous	
2	.....	.....	Intraperitoneal	
1	....	.....	Intravenous	

immunity to pneumococcus type 1 in rabbits by vaccination through the buccal mucous membrane.

In these two preceeding experiments an attempt had been made to immunize 30 rabbits and tests with massive infecting doses showed that success had been obtained with 23 animals. They gave marked evidence of generalized immunity. Of the 7 rabbits which failed to survive there is some question whether 3 of these died as a direct result of the test infecting dose.

Furthermore, in reference to table 3 none of the immunized rabbits which died did so before the third day. One died on the third day, two on the fifth, one on the sixth, and one on the seventh day. All 9 controls died within 12 hours.

Four months after immunizing these rabbits 19 of them were again tested for their immunity with exactly the same method as was used 10 days after vaccination when they were given their first test injection. The results in table 4 show that 13 were still immune and able to withstand test doses of virulent pneumococci which killed nonimmune rabbits in 12 to 24 hours.

CONCLUSIONS

Rabbits die quickly of pneumococcus septicemia when type 1 pneumococci are introduced through a scratch in the buccal mucosa of the cheek.

Evidence is advanced showing that it is comparatively easy to stimulate the production of generalized immunity to the pneumococcus, type 1, in the rabbit by introducing a killed pneumococcus vaccine just beneath the buccal mucosa of the cheek.

A surprisingly small amount of vaccine is necessary for immunization, one minim on each of 10 successive days being sufficient.

At the present time this immunity has persisted for 4 months.

All attempts to immunize subcutaneously and intradermally by injecting 1 minim on 10 successive days, by instillation of 1 minim into the eye, or by swabbing the nose and mouth have failed to result in any protective immunity.

STUDIES ON ANTHRAX IMMUNITY

II. THE IMMUNIZATION OF SHEEP BY MEANS OF ANTHRAX BACILLI ATTENUATED WITH SODIUM CHLORIDE

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A previous publication[1] has described a method of attenuating cultures of B. anthracis to an extent which permits of their use as a vaccine on rabbits. The method of attenuation consists of growing anthrax bacilli through a series of six subcultures made at weekly intervals on agar slants; the agar used is of the same composition as standard agar, except that it contains 5% NaCl. The sixth subculture of virulent B. anthracis grown on such media was found to be practically avirulent for guinea pigs and rabbits. A group of four rabbits inoculated with two successive doses of the attenuated culture withstood, 14 days later, an injection of virulent anthrax bacilli; the same dosage of virulent bacilli proved fatal in 22 to 40 hours for control animals. This paper presents results secured in using the same strain of attenuated culture as a vaccine for sheep.

PREPARATION OF VACCINE

The attenuation of the original NaCl attenuated cultures was completed May 21, 1925. A standard agar slant was inoculated from the original cultures May 27, and transferred to fresh standard agar slants June 24; from the latter it was transferred to Dunham's peptone broth July 28, and from there to flasks containing agar July 29. The growth appearing in the agar flasks served as the vaccine used in the work herein reported. The number of transfers of the attenuated culture is cited as a check on the stability of the characteristics of attenuation and antigenic efficacy possessed by the culture in question.

After four days' incubation of the inoculated flasks at 37 C. the growth was washed off with sterile physiological salt solution, sterile glass beads were added to the suspension, and the whole was subjected to 15 minutes of vigorous rotary motion by hand. The suspension was then filtered through glass wool and enough 5% phenol was added to it to produce a concentration of 0.5% phenol. The suspension of attenuated culture corresponded in density to tube 3 of the McFarland nephelometer. In grinding a bacterial suspension with glass beads, many small particles of glass become detached and go into suspension. Therefore, the actual number of bacilli contained in the material here under consideration was far less than would be indicated by the nephelometer reading. A bacterial count by the plating method was attempted; five replications of agar plates, each containing 1 cc. of a 1-40,000 dilution of the original suspension were prepared. The number

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[1] Schilling, S. J.: J. Infect. Dis., 1926, 38, p. 341.

of colonies counted on the plates at 48 and 96 hours varied from 0 to 27; therefore the count was not considered of practical significance.

INOCULATION OF SHEEP WITH ATTENUATED CULTURES

One hour after the preparation of the suspension, four sheep were inoculated subcutaneously with this material.

The injections were made in quantities of 1, 2, 3 and 5 cc. (table 1). The site of inoculation in each case was the inside of the right foreleg, where there is no wool and where on this account close observation of local reaction can be made. In order to observe the appearance of any general reaction, the temperature of all inoculated sheep and of two controls was taken at the time of inoculation and every 24 hours thereafter for 14 days. The range of normal temperatures in sheep is wide; some authorities [2] give the normal range as 102.2-104.9 F.; others [3] as 101.3-105.8 F. The latter probably is more nearly correct, since the variation found among the control sheep during the period of observation was from 102.7 to 106.1 F.

TABLE 1

INOCULATIONS OF SHEEP WITH NaCl ATTENUATED AND VIRULENT B. ANTHRACIS

Sheep	Cc. of NaCl Attenuated Culture Subcutaneously 8/2/25	Cc. of Virulent Culture Subcutaneously 10/7/25	Results
1	1.0	2.0	Marked general and local reactions. Complete recovery
2	2.0	2.0	
3	3.0	2.0	
4	5.0	2.0	
5	—	2.0	Controls, died, 54 hours
6	—	2.0	

A study of the temperature records does not indicate the occurrence of a general reaction from the effects of inoculation. Furthermore, there was no apparent correlation between the quantity of bacterial suspension and the subsequent temperature of the animals.

Local reactions occurred in all inoculated animals; they took the form of small swollen areas which rapidly became more firm and diminished in size, although they could be felt for a period of 16 days. Sheep 1 developed less local reaction than the others; the degree of local reaction occurring in sheep 2, and 3 seemed to be about equal, and sheep 4 presented the most marked reaction, which was in accordance with the relative amounts of vaccine administered to them. In the latter case an edematous area 3 inches long and 1½ inches broad and about ¼ inch in thickness developed by the third day. This area was soft to

[2] Malkmus, Bernard: Outlines of Clinical Diagnosis, 1913, p. 65.
[3] Henry, W. A., and Morrison, F. B.: Feeds and Feeding, 1923, p. 57.

the touch; it seemed to remain unchanged for several days and then gradually became smaller and more firm. Sheep 1, 2, and 3 did not develop an edematous swelling; a rather firm tumefaction about the size of the end of a thumb, however, did appear. Lameness did not occur in any animal at any time, nor were there any indications of impairment of appetite or general well-being.

INOCULATION OF SHEEP WITH VIRULENT CULTURES

Eleven weeks after administration of attenuated cultures to the sheep, they were inoculated subcutaneously with 2 cc. each of a suspension of virulent anthrax bacilli (table 1). Two control animals were similarly infected. The site of inoculation in each instance was the inside of the left forearm. This site was chosen in order that it might not coincide with the site of vaccination, which was the inside of the right forearm.

The suspension of virulent cultures used in inoculating the sheep was prepared by inoculating a quantity of standard broth heavily with spores from a known virulent strain. After 24 hours incubation, sterile glass beads were added to the culture and the flask containing this material was subjected to a rotary motion for ten minutes. The suspension of bacteria in broth was then filtered through glass wool and was immediately used. When compared with the McFarland nephelometer, the suspension was found to be slightly more dense than tube 1 and somewhat less dense than tube 2 of that standard. A bacterial count of the suspension by the agar plate method yielded close agreement in five replications. Two cc. of a 1-100,000 dilution of the suspension yielded 26, 26, 25, 33, and 35 colonies on the five agar plates, or an average of 29.2 colonies per plate. From these plates the bacterial count of the suspension appeared to be about 1,460,000 bacilli per cc. Since many of the colonies undoubtedly developed from chains of two or more elements, the actual number of bacilli per cc. probably was much higher.

Temperatures were taken of all sheep every six hours during the time when fatalities were expected, and three times daily after this until the temperatures of the vaccinated animals became normal. There was a marked rise in the temperatures of all animals. In the control animals it was somewhat deferred, and not so high as that of the vaccinated sheep, and at the time of death their temperatures were well within the limits of normal variation. Sheep 1 presented a series of remissions and exacerbations of temperature; these should be interpreted as being caused by fully as serious a disturbance of the thermal regulatory

centers of the nervous system as though a constant abnormally high temperature had occurred during the same period. Sheep 2 carried an abnormally high temperature longer than any of the others. Sheep 1, and 2 probably underwent a more severe general reaction than 3 and 4; it may be significant that this coincides with the fact that sheep 1 and 2 had received a smaller dosage of vaccine than sheep 3 and 4.

The local reactions to the inoculations with virulent bacilli were well marked in all cases. Twenty-four hours after inoculation all of the animals were lame and tender to pressure over the site of the injection; very little swelling of adjacent tissues could, however, be noted and this was of a diffuse character. Forty-eight hours after inoculation the local reactions on control animals 5 and 6 presented marked enlargement and were much softer to the touch than those on vaccinated animals; there was evidence of edema between the skin and fascia which caused sacculation of the former structure. At this time the vaccinated animals presented rather firm swellings about as large as the end of a thumb, which became smaller and harder from this time until they disappeared.

The two control animals died about 54 hours after inoculation. On postmortem the findings on both of them were those of typical anthrax. The blood was uncoagulated and the spleens were greatly enlarged. Capsulated bacilli in large numbers appeared in microscopic preparations from the blood and spleen pulp. Cultures were isolated from the heart blood which were typical of B. anthracis and inoculation of these cultures into guinea pigs produced death from typical anthrax; the heart blood of the guinea pigs also yielded pure cultures of anthrax bacilli.

During the period when the temperatures of the sheep were high they all showed marked acceleration of rate of respiration. No evidences of illness other than high temperature, rapid respiration, and a certain degree of cyanosis were apparent at any time, however, on observation of vaccinated or control animals. Previous to the inoculation with virulent cultures the sheep were kept on pasture during the day and were brought into a barn each night. At this time they were fed some oats, and had available hay which, however, they refused to eat. When the sheep were about to be inoculated with the virulent culture they were confined in the barn and kept there continuously during the test; the only feed they consumed while they were confined was whole oats. Forty-eight hours after the administration of the virulent culture, however, the sheep refused to eat the oats, which fact was at the time interpreted as a sign of inappetence. Yet they persisted in refusing feed after their temperatures became normal and when they were returned

to pasture on the fourth day after inoculation they fed eagerly. Thus their refusal of grain probably was no symptom of illness, but rather the result of being deprived of their usual rations.

Ten days after inoculation of the sheep with virulent cultures it was found that a swelling about the size of the end of a small finger persisted at the site of inoculation on sheep 2. A skin incision was made and agar cultures prepared from the swollen tissue; this tissue was found to be firm and apparently was fibrous in character. The cultures secured were found on incubation to be anthrax bacilli. A guinea-pig was inoculated with this culture by making a small incision in the skin of the abdomen and applying a loopful of the culture. The guinea-pig was found dead on the morning of the third day and postmortem examination, as well as cultures prepared from the heart blood, proved that it had died of anthrax. Thus it appears that virulent anthrax bacilli may be harbored by infected animals for some time at the site of inoculation.

DISCUSSION

The number of animals utilized in this test of the efficacy of NaCl attenuated anthrax bacilli when used as a vaccine manifestly is small. The results on this small number, however, are clear cut; the vaccinated animals surived later infection with virulent bacilli in quantities which caused the death of the control animals of typical anthrax. It is further to be noted that but one injection of vaccine was given, and thus a severe test of its potency was made. In field practice in this country, three or four injections of commercial (modified Pasteur) vaccine are given. The successive inoculations are made with vaccines of graded virulence and 14 to 21 days are permitted to elapse between administrations. This is found in practice to result in a higher degree of resistance to infection than the administration of a single dose of vaccine. Since the sheep utilized in this experiment were found resistant to heavy infection after a single administration of the NaCl attenuated vaccine, it may be expected that a repeated administration of vaccine would lead to a higher degree of protection, and their survival may be considered to be distinctly significant. The immunity resulting from the administration of the NaCl attenuated culture was patently not a local, but rather a general immunity. Care was taken in all instances to administer the vaccine on the inside of the right forearm and later the virulent culture at a similar location on the left leg.

The absence of any perceptible general reaction and the appearance of only a mild local reaction at the site of inoculation which were noted upon administration of the vaccine are indicative of the low degree of pathogenicity of the NaCl attenuated culture. They bear out the observations made upon inoculated guinea pigs and rabbits reported in

a previous paper.[1] The characteristic of extremely low virulence seems to be coupled with the characteristic of high antigenic efficacy in the NaCl attenuated culture. Further work on larger numbers of subjects which should also include other species of animals will be necessary to more completely elucidate this point. Experiments with varying doses and means of administration of vaccine will also be necessary in this connection. This work is in part under way and will be pursued as rapidly as circumstances permit.

Some little evidence was gained incidentally to the work herein presented on the stability of the degree of virulence and antigenic qualities possessed by the NaCl attenuated culture upon cultivation on ordinary bacteriological media. If the attenuated culture would tend rapidly to reacquire a higher degree of pathogenicity upon such cultivation, or if on the other hand it should rapidly lose its antigenic qualities, its practical value would be questionable. The records herewith presented show that this culture appears to have maintained its antigenic efficiency after four transfers involving standard agar and Dunham's peptone broth during a period of over two months. More recent evidence, which will be detailed elsewhere, also substantiates the view that the NaCl attenuated culture is rather constant in both its reduced pathogenicity and its antigenic value. Moreover, the work of Bail[4] gives strong evidence that a return to pathogenicity should not be expected to occur. Bail experimented on increasing the virulence of an attenuated (non-capsulated) strain of B. anthracis by repeated passage through guinea pigs and met with negative results. It would more readily be possible that prolonged cultivation on artificial media would cause impairment of the value of the NaCl attenuated culture by causing loss of antigenic properties, since this occurs when dealing with other species of bacteria, than that virulence would be regained.

A point may be raised regarding the actual virulence of the "virulent culture" used in this experiment. All laboratory workers recognize that there are different degrees of pathogenicity among virulent strains of anthrax bacilli. The original source of the virulent culture used in this experiment is not known. However, in several years of laboratory experience with it, it has been found regularly to kill guinea pigs and rabbits in 20 to 48 hours upon subcutaneous administration. Furthermore, this strain of B. anthracis killed sheep in 54 hours upon subcutaneous inoculation in this experiment, and thus may be regarded as a

[4] Centralbl. f. Bakteriol., I. O., 1914, 75, p. 159.

rather virulent culture. Further experimental work on the NaCl attenuated vaccine will also take this point into consideration and will provide for the administration of other strains of virulent cultures to the vaccinated animals.

CONCLUSIONS

A single injection of a sodium chloride attenuated culture of B. anthracis protected sheep against subsequent infection with a virulent culture; the control animals died in 54 hours of typical anthrax. The immunity conferred by the vaccine was general. Vaccination and subsequent inoculation with virulent cultures were performed subcutaneously but in widely separated sites of administration.

Virulent anthrax bacilli may be found at foci of previous infection for some time following the apparent recovery of the infected animal.

The reduced pathogenicity as well as the antigenic properties of the sodium chloride attenuated culture of B. anthracis appeared to remain fairly constant during cultivation on artificial medium.

CELLOBIOSE FERMENTATION BY COLI-AEROGENES GROUP

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Several procedures are now available for differentiating Bacterium coli of intestinal origin from other closely related forms which seem to have a natural habitat in soil. These methods have aroused considerable discussion regarding the desirability of their practical use in the sanitary examination of water, and it is safe to say that at the present time there is no uniformity of opinion on this point. It is to be expected, therefore, that any new method for differentiating these types should command some interest, since it may serve to simplify the procedure of differentiation or to clear up some of the difficulties in interpreting results of the laboratory examination.

The use of cellobiose for the differentiation has been suggested recently by Jones [1] who found that in a series of 53 cultures of the coli-aerogenes group isolated from feces, soil, and water, the Bacterium coli type did not attack cellobiose, while all strains of Bacterium aerogenes fermented it with abundant gas formation. The former methods which have been suggested for this separation have been based upon other characteristics such as dextrose metabolism, the methyl red and Voges-Proskauer tests; or the ability to use certain simple compounds of nitrogen or carbon when supplied in chemically definite mediums, the uric acid and citrate mediums. The new method suggested by Jones depends upon the qualitative fermentation of a sugar, and the test can be made according to the usually accepted methods of determining fermentation.

The object of the present investigation has been to review the fermentation of cellobiose by the coli-aerogenes group and to ascertain its correlation both with the source of the cultures and with the results of other differential tests. Advantage was taken of a rather large collection of coli-aerogenes cultures isolated by the writer from a variety of sources. Some of these cultures were isolated recently, either from feces or soil, while others from similar sources had been kept in stock for two years or more.

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[1] Science, 1924, 60, p. 455; Abstr. Bact., 1925, 9, p. 5.

Since cellobiose is an uncommon sugar and has not been used in bacteriological work, it deserves a brief explanation regarding its structure. It is a disaccharide and bears much the same relationship to cellulose that maltose does to starch. It reduces Fehling's solution. On hydrolytic cleavage it gives rise to two molecules of glucose. It is considered to be a 5-glucose β glucoside.[2]

The differential tests employed to place the cultures in their respective sections and to compare with the fermentation of cellobiose were the methyl red and Voges-Proskauer tests and the citrate test. The uric acid test was omitted since in the writer's experience [3] it does not correlate as closely with the source of the cultures as does the citrate test, and in addition, it is less satisfactory for general use. The technic of performing the differential tests was similar to that previously used.[3] Cultures for the methyl red and Voges-Proskauer tests were grown in the standard broth [4] consisting of 0.5% each of dextrose, Witte peptone and dipotassium hydrogen phosphate for 4 days at 30 C. An incubation period of 3 days at 37 C. was used also. For the citrate test the synthetic citrate medium was employed as described in earlier publications.[5] Observations of growth were made after 3 days at 30 C. Cultures giving a negative test at this time, i. e., showing no growth, were held for several weeks longer and examined at intervals to detect any delayed utilization of the citrate.

The procedure used for testing the ability to ferment cellobiose departed somewhat from the usual methods of determining sugar fermentation. Cellobiose is expensive and obtainable only in small quantities and for this reason tests were not made in the ordinary large Durham or Smith fermentation tubes, but a special method was adopted by which fermentation could be detected in small amounts of medium. A standard meat extract peptone medium plus 0.5% agar was made and to this the indicator, brom-thymol-blue, was added in the amount suggested by Baker.[6] Just before tubing, cellobiose was added to give a 0.5% concentration and the resulting medium was then put, in 2 to 3 cc. amounts, into small test tubes of 10 to 11 mm. diameter, and sterilized in the autoclave. Inoculations were made by stabbing the soft agar medium with the straight wire. Acid production was detected by the characteristic color change of the indicator and gas by the torn appear-

[2] Haworth and Hirst: J. Chem. Soc., 1921, 119, p. 193. Armstrong, E. Frankland: The Simple Carbohydrates and the Glucosides, 1919.
[3] Koser, S. A.: Jour. Bact., 1924, 9, p. 59; J. Infect. Dis., 1924, 35, p. 14.
[4] Standard Methods of Water Analysis, Am. Public Health A., 1925.
[5] Koser, S. A.: J. Bact., 1924, 9, p. 59; J. Am. Water Works A., 1924, 12, p. 200.
[6] J. Bact., 1922, 7, p. 301.

ance of the agar medium. Usually gas was produced promptly and in sufficient amount to disrupt or tear markedly the soft agar medium. As a routine procedure fermentation was recorded after incubation at 30 C. for 48 hours. Cultures negative at this time were examined from time to time for a period of 3 weeks to note appearance of any delayed fermentation. A number of cultures were also run at 37 with results similar to those secured at 30, though the limited amount of cellobiose forbade testing the entire series of cultures at both temperatures.

The source of the cultures and results of the differential tests are shown in table 1. Bacterium coli, whether derived from man or certain of the lower animals, is unable to ferment cellobiose while Bacterium

TABLE 1

Differential Tests of Cultures from Different Sources

Source	Section of Group	Number of Cultures	Differential Tests			
			MR	V-P	Citrate	Fermentation of Cellobiose
Feces, human	Bacterium coli	43	+	0	0	0
Feces, animal	Bacterium coli	33	+	0	0	0
Feces, human and animal	Bacterium aerogenes	3	0	+	+	Acid and gas
Soil, either free from pollution, or exposed to chance contamination	Aerogenes-cloacae	104	0	+	+	Acid and gas; 103 Acid; 1
	Irregular	11	Irregular		mostly +	Acid and gas; 10 0; 1
	"Intermediate" type	29	+	0	+	Acid and gas; 24 Acid; 3 0; 2
Soil, from cultivated fields	Apparently Bacterium coli	18	+	0	0	Acid and gas; 11 0; 7

aerogenes ferments it readily. This substantiates the work of Jones.[1] The few aerogenes cultures isolated from feces fermented cellobiose promptly and thus agreed with the aerogenes obtained from soil. In so far as the above two well defined types are concerned there appears to be an almost perfect correlation of tests. Some cultures, however, do not fall into either one of these subdivisions. For example, 11 cultures gave irregular results with the methyl red and Voges-Proskauer tests. In cultural characteristics and deportment they resembled the aerogenes-cloacae section, though the results of repeated methyl red and Voges-Proskauer tests were not clear cut. Nine of these cultures developed readily in the citrate medium, one grew slowly and the other was negative. It is noteworthy that ten of them fermented cellobiose promptly and could thus be separated from the Bacterium coli type.

The significance of the so-called "intermediate" type has been discussed elsewhere.[5] Suffice it to say here that organisms may be found in soil, which resemble intestinal Bacterium coli in their department toward the methyl red and Voges-Proskauer tests. However, these soil organisms are usually citrate positive, in contrast to the intestinal coli which fails to utilize citrate. It would seem that these soil forms should not be confused with the true Bacterium coli of intestinal origin, so they have been tentatively designated as the "intermediate" type or section. It is interesting to see that most of these cultures fermented cellobiose promptly, and may be distinguished from the intestinal coli by this means as well as by the utilization of citrate. This would seem to be additional evidence that the writer's separation of the "intermediate" cultures from the true Bacterium coli is a valid one.

Finally, among the cultures obtained from soil, there were 18 which resembled in a general way the true Bacterium coli. All of these cultures were isolated from the soil of cultivated fields exposed to chance contamination and it is rather to be expected that at least a few intestinal coli would be found under these conditions. All 18 cultures were similar to Bacterium coli on the basis of the methyl red, Voges-Proskauer, and citrate tests. On the basis of cellobiose fermentation 7 resembled coli, while the remaining 11 produced fermentation promptly. The question arises whether these 11 cultures are really intestinal coli which contrary to general rule fermented cellobiose, or whether they are in reality soil forms related perhaps to the "intermediate" type, even though failing to utilize citrate. Certain cultural features point to the latter conclusion. Most of them produced a light yellow pigment on agar slants, especially in older cultures; they also exhibited delayed lactose fermentation and gave rise to colonies on Endo medium unlike those formed by the true Bacterium coli. These features taken in connection with the positive cellobiose tests indicate that these 11 cultures should be set apart from the intestinal type. Similar types were not encountered in feces and it seems probable that they are normal soil forms. Here the fermentation of cellobiose has been more useful than the other tests in differentiating these cultures from the intestinal type.

It should be stated that even among the apparently typical coli or aerogenes types a few variations from the accepted behavior may be encountered. Thus, 3 of the 33 coli cultures from animal feces showed a slow growth in the citrate medium and produced a visible turbidity usually after five or six days. Another culture from the same source exhibited delayed fermentation of cellobiose, forming acid but no gas

after 7 days. However, these occasional irregularities did not interfere with the differential tests as they were not apparent after the two or three day interval when the results are usually recorded. They were detected in the present investigation only because all cultures were held for an additional period beyond that ordinarily used.

SUMMARY

On reviewing the entire collection of cultures it is evident that insofar as the two well defined sections of the group are concerned there appears to be an almost perfect correlation of tests, the intestinal Bacterium coli is quite distinct from the well known aerogenes-cloacae forms. When we consider certain other members of the group obtained from soil, especially the "intermediate" type and the irregular forms, the same striking correlation between all of the tests is not apparent. There is, however, a marked correlation between source and the results of the citrate and cellobiose tests. These tests appear to be more significant than the methyl red and Voges-Proskauer tests in indicating the source of the cultures. Again the point is emphasized, as in previous work,[3, 5] that we are not justified in assigning a fecal origin to all methyl red positive cultures which may be encountered in soil or other sources outside of the animal body.

A METHOD OF INCREASING THE VIRULENCE OF CLOSTRIDIUM CHAUVOEI BY THE USE OF FERRIC SALTS

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For some years investigations have been made on the use of various substances, including different peptones, in the preparation of standard brain-liver medium upon the virulence of Clostridium chauvoei. The basic medium contains equal parts of brain pulp and liver broth, 1% peptone and 0.5% salt.

Strains of Cl. chauvoei isolated from cattle sometimes maintained their virulence for many years, then suddenly became avirulent; other strains retained their virulence for only short periods; and a few strains were avirulent almost from the time of isolation. Varying the composition of the medium by the use of different peptones and the addition of carbohydrates together with transferring at frequent intervals did not increase the virulence of the cultures.

In the spring of 1925 it was noted that a culture of Cl. chauvoei previously obtained from Professor I. C. Hall of the University of California was considerably less virulent than a subculture of the same strain obtained from him at that time.

About that time an article by Otto Warburg [1] "Iron the oxygen-carrier of respiration-ferment" appeared. This article suggested the use of iron salts for the stimulation of the cellular activities of Cl. chauvoei. It was hoped that their use would result in an increased production of the so-called "lethal substance," [2] and thus increase the virulence of the cultures.

Avirulent strains of Cl. chauvoei were grown by transferring three to five times at intervals of two to three days in medium containing 0.2% of either ferric sulfate, ferric chloride, or ferrous sulfate, after which the cultures were tested for virulence on guinea pigs. Avirulent strains grown on the basic brain-liver medium or in medium containing ferrous sulfate remained avirulent while the same strains grown in medium containing ferric salts rapidly increased in virulence. One

Received for publication, Feb. 20, 1926.

[1] Science, 1925, 61, p. 575.

[2] Scott, J. P.: J. Bact., 1925, 10, p. 300.

strain which was used regularly between 1917 and 1921 for the production of blackleg in calves for the manufacture of blackleg aggressin, but which had lost its virulence, was in this way raised in virulence so that it again killed guinea pigs and calves. Strains of Cl. oedematis and Cl. novyi were also increased in virulence by passage through ferric sulfate medium. Passage of the newly virulent strains through medium containing no ferric salts or containing ferrous sulfate resulted in a loss of virulence.

TABLE 1

Virulence of Cultures Grown in Medium Containing Iron Salts

	Brain-Liver	Hall Medium	Brain-Liver 0.2%, Ferric Sulfate	Hall Medium 0.2%, Ferric Sulfate	Brain-Liver or Hall 0.2%, Ferric Chloride	Brain-Liver or Hall 0.2%, Ferrous Sulfate
Total number guinea-pigs inoculated	7	4	9	25	15	15
% guinea-pigs dead or having marked lesions	0	25	66	84	46.6	0

TABLE 2

Action of Iron Salts on Fermentative Powers of Anaerobes

Enlows' Medium with Peptone or Iron Salts as Indicated

	Cl. chauvoei				Cl. oedematis				Cl. novyi			
	0.5 % Peptone	0.2 % Ferrous Sulfate	1% Peptone	1% Peptone 0.2% Ferrous Sulfate	0.5 % Peptone	0.2 % Ferrous Sulfate	1% Peptone	1% Peptone 0.2% Ferrous Sulfate	0.5 % Peptone	0.2 % Ferrous Sulfate	1% Peptone	1% Peptone 0.2% Ferrous Sulfate
Glucose...	0	a g	a 0	A G	a g	A G	A G	A G	0	0	A g	A G
Glycerol..	0	0	0	0	0	a 0	a g	a g	0	0	0	a 0
Lactose...	a g	a g	a g	A G	A g	A G	A G	A G	0	0	0	0
Mannose..	0	0	0	0	0	0	a g	a g	0	0	0	0
Salicin....	0	0	0	0	A G	A G	A G	A G	0	0	0	0
Sucrose...	0	A g	A G	a 0	0	0	0	0	0	0	0	0
Control...	0	0	0	0	0	0	a g	a g	0	0	0	0

A G and a g indicate marked and slight acid and gas production, respectively.

Strains passed through guinea pigs for one to seven passages were not increased in virulence as regularly as when passed through ferric sulfate medium.

Table 1 shows the great increase in virulence produced by ferric sulfate especially when added to Hall's brain-liver medium, containing 2% peptone, 0.1% glucose, and no sodium chloride.

Iron salts added to Enlows' synthetic medium [3] stimulated carbohydrate fermentation especially if the amount of peptone was increased. The fermentative powers of Cl. chauvoei, Cl. oedematis and Cl. novyi in these mediums are shown in table 2.

[3] Pub. Health Reports, 1923, 38, p. 2129.

Filtrates of three and ten day cultures of Cl. chauvoei grown in media containing ferric sulfate or ferric chloride were shown to be non-toxic to guinea pigs in doses of 2 cc. intracardially, 4 cc. intraperitoneally, and 7 cc. subcutaneously. The aggressive action of these filtrates was the same as that of filtrates produced from cultures grown in iron-free medium.

No correlation between the virulence and the fermentative activities of the strains could be found. Nor could any correlation be noted between virulence and antigenicity.

It is believed that these results tend to confirm the presence of a lethal or specific cellular substance in the cells of virulent cultures of Cl. chauvoei. It is also believed that the presence or absence of such a substance determines whether the culture shall be virulent or avirulent, and possibly constitutes the only difference between virulent and avirulent cultures.

The addition of 0.2% sodium citrate to medium containing ferric sulfate has been shown to increase the amount of soluble iron in the medium and prevents the precipitation of phosphates.[4]

Recently Welo and Baudish [5] in studying the catalytic actions of certain oxides of iron find that a ferric oxide when added to culture medium will maintain the virulence of Bacterium lepisepticum while iron-free medium will not.

SUMMARY

Experiments with many strains of Clostridium chauvoei have shown the difficulty of maintaining the virulence of these organisms.

The addition of ferric sulfate to culture media stimulated these organisms to such an extent that avirulent strains regained their virulence.

The virulence of Cl. chauvoei is independent of the fermentative or antigenic properties.

These results are thought to confirm the presence of the so called "lethal substance" or specific cellular activity of virulent cultures of Cl. chauvoei.

[4] Hopkins, E. F., and Wann, F. B.: J. Gen. Physiol., 1925, 9, p. 205.
[5] Science, 1925, 62, p. 311.

THE INCIDENCE OF PNEUMOCOCCUS TYPES IN PNEUMONIAS OF CHILDREN

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The bacteriology of pneumonias in children has not been studied as extensively as that of the pneumonias in adults, in which the types of pneumococci and their incidence have been well established by the work of Dochez and Gillespie,[1] Cole,[2] Avery, Chickering, Cole and Dochez,[3] Avery,[4] Cecil and Larsen,[5] and others. The literature relating to the pneumonias of children has dealt with the clinical findings and treatment rather than with the etiologic agent.

Lyon[6] in a study of 98 cases of lobar pneumonia in children found the following percentages: type 1, 29.9%; type 2, 3.0% (including atypical); type 3, 7.1%; type 4, 37.7%. These findings corresponded closely with those found by him in a parallel series of cases in adults. The source of the pneumococci included the blood, lung puncture, pleural fluid and sputum. In the sputum from 73 cases of lobar pneumonia in infants and children up to 12 years of age, he found the following percentages: type 1, 19.1%; type 2, 2.7%; 2a, 1.4%; type 3, 9.6%; type 4, 48%; other organisms 9.6%; no results were obtained in 9.6%. This worker found a discrepancy in the sputum in only three cases as compared with the blood, lung or pleural fluid.

In 31 cases of bronchopneumonia Lyon found the following types in the sputum: type 1, 9.7%; type 2, 3.2%; type 2, atypical, 3.2%; type 3, 6.4%; type 4, 38.7%. He noted a decrease as compared with lobar pneumonia, in the total occurrence of fixed types, the reduction being largely at the expense of type 1. The increase in the occurrence of group 4 he considered relative rather than absolute.

According to Lyon the relative mildness of lobar pneumonia in childhood can be explained by one of two hypotheses. First that the bulk of cases are caused by the less virulent strains of pneumococci such as group 4, or second, that the incidence of fixed types being much as for adults, the child opposes to them a better natural resistance than the average adult. He made the statement that "experiments have led to the statement that Group IV pneumococci are largely responsible for pneumonias of infancy and childhood and that the fixed types are relatively infrequent, Type I being comparatively rare."

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[1] J. Am. M. A., 1913, 61, p. 727.
[2] N. Y. M. J., 1915, 101, p. 1.
[3] Monograph No. 7, Rockefeller Inst. for Medical Research, 1917.
[4] J. Am. M. A., 1908, 70, p. 17.
[5] Ibid., 1922, 79, p. 343.
[6] Am. J. Dis. Children, 1922, 23, p. 72.

Webster[7] studied the frequency of pneumococcus types in children in Australia. He also found that the distribution in children followed closely that observed for adults in the same center. In 147 cases of lobar pneumonia in adults he found the following distribution of types of pneumococci: type 1, 43%; type 2, 9.5%; type 3, 4.7%; heterogenous, 34.7% (corresponding to the American group 4). In 120 cases of lobar pneumonia in children the following distribution was noted: type 1, 38.3%; type 2, 5.0%; type 3, 5.8%; heterogenous, 40.8%. The Australian type 4, distinct from the American group 4, was identified in a small number of cases.

In a study of 48 cases of lobar and broncho-pneumonias in children under six years of age Pisek and Pease[8] found the distribution as follows: type 1, 22.9%; type 2, 29.3%; type 3, 8.3%; type 4, 39.8%.

Mitchell[9] states that the "fixed types are infrequent in infants and children as compared with adults." He found pneumococci of group 4 to occur most frequently. Of 90 children examined, 11.1% showed the presence of type 1; 11.1%, type 2; 3.3%, type 3; 74.4%, group 4. Hartley[10] also considered group 4 the one most frequently met with in children.

A direct comparison of the above figures with those which have been determined for the pneumococcus pneumonias of adults is not possible, because the variations in type incidence in adults have usually been given in terms of pneumococcus pneumonia only, whereas in the above reports relating to children, with the exception of those of Mitchell and of Pisek and Pease, the percentages of pneumococcus types are given in terms of all the pneumonias investigated. If the above percentage variations are recalculated in terms of the pneumococcus pneumonias, then the incidence of the various types of pneumococci, in 401 cases of pneumococcus pneumonia included in the 460 cases of pneumonia reported above, is as follows: type 1, 16.0 to 42.0%, average 26.0%; type 2, 4.0 to 29.0%, average 11.0%; type 3, 3.0 to 12.0%, average 8.0%; group 4, 40.0 to 74.0%, average 55.0%. These figures may be compared with the combined statistics of Dochez and Gillespie,[1] Cole,[2] Avery, Chickering, Cole and Dochez,[3] Avery,[4] and Cecil and Larsen,[5] based on a total of 1,552 cases of pneumococcus pneumonia; type 1, 33.0 to 41.0%, average 36.0%; type 2, 18.0 to 34.0%, average 28.0%; type 3, 7.0 to 17.0%, average 13.0%; group 4, 20.0 to 33.0%, average 23.0%. The variations in type incidence, based upon the above average figures, may be tabulated as follows to render comparison easier:

	Adults (1,552 Cases), %	Children (401 Cases), %
Type 1	36.0	26.0
Type 2	28.0	11.0
Type 3	13.0	8.0
Group 4	23.0	55.0

Previous work indicates a considerably higher incidence of group 4 strains in the pneumonias of children as compared with those of adults, this increase being associated with a fairly even decrease in the remaining three types.

[7] M. J. Australia, 1924, 1, p. 129.
[8] J. M. Sc., 1916, 151, p. 14.
[9] Pennsylvania M. J., 1917, 20, p. 343.
[10] Virginia M. Monthly, 1922, 49, p. 430.

INVESTIGATION

The following work was undertaken with the view of determining the types of pneumococci present in the throats of children ill with pneumonia, and of comparing this incidence with that of the pneumococci found in the throats of children with diseases other than pneumonia. It was thought that the type of pneumococcus found in the throat early in the disease would be a fairly reliable index of the type causing the infection. While the sputum method is perhaps the best to use in typing pneumococci, it is not very practicable when dealing with children. Kelley and Gussin,[11] in a series of 136 cases of lobar pneumonia in adults, obtained practically the same results from throat swabs as when the sputum was used.

Material.—During the period from January, 1924, to August, 1925, 103 cases of pneumonia and 48 of other diseases occurring in children between the ages of 3 weeks to 11 years, were studied in the wards of Michael Reese and Cook County Hospitals (Chicago). Thirteen other respiratory diseases, thought at first to be pneumonia, have been classified separately. The number of pneumonias studied represents about ¼ of the number of such cases occurring in these hospitals during that period of time. The cultures were made during the first week of the disease, whenever possible.

Experimental Procedure.—Mouse inoculation was carried out for the most part, as well as inoculation of blood agar plates. One c c. of a six hour blood broth culture (blood cells allowed to settle to the bottom of tube) was inoculated intraperitoneally into a white mouse. At the same time blood agar plates were streaked from the broth cultures. After 24 to 48 hours incubation the plates were examined for pneumococcus-like colonies and an average of six colonies picked to other blood plates. The mice were allowed to die and were necropsied as soon as possible after death. If the mouse did not die within 14 days it was killed and cultures made from the peritoneal fluid and heart's blood. In no case was the pneumococcus recovered from the killed mice, although in several instances the plate culture showed the presence of pneumococci.

It was quite interesting to note that in many instances the mice did not die from the injection for several days. In one case of lobar pneumonia, a type 1 pneumococcus was recovered from the mouse 10 days after injection. Organisms of group 4 were often recovered 2 to 7 days after injection.

Agglutination and bile solubility tests were carried out on the isolated cultures, veal infusion broth, P_H 7.8, being used in all cases Although a heavier growth could be obtained when dextrose was added to the broth, it was found that the bile tests were more satisfactorily carried out without the presence of sugar. The antipneumococcus serum was obtained from the Division of Laboratories and Research of the New York State Department of Health. This serum was used as indicated on the containers, namely, types 1 and 2, undiluted and 1 : 10, type 3, undiluted and 1 : 5. The tests were incubated at 37 C for two hours, read, then placed in the icebox and read again after 18 to 24 hours.

[11] J. Infect. Dis., 1924, 35, p. 323.

Whole untreated bile was used for testing the solubility of the pneumococci. This was used in the proportion of 1:2. Solution took place, as a rule, within a few minutes. Several insoluble and partially soluble strains were encountered. No special study, however, was made of these strains.

Sugar fermentation tests were not used in the differentiation of the pneumococci. Inulin fermentation as a diagnostic test was found to be unreliable and was discarded.

DISCUSSION

The similarity between the types found in lobar and those found in bronchopneumonia is quite striking (table 1). The incidence of type 2, however, is higher in this series in lobar pneumonia than in bronchopneumonia. Since there were twice as many cases of lobar as bronchopneumonia, the slight differences in pneumococcus incidence in the two groups does not permit any definite conclusions. A sharp clinical differentiation between the two forms of pneumonia in children is not always possible, and the predominance in the early years of childhood of bronchopneumonia over lobar pneumonia, due to the pneumococcus, has been noted. There is a noticeable scarcity of type 1 which, in adults, usually shows a higher percentage. Webster [7] and others have noted seasonable variations of the various types, especially of type 1. This might be a factor in the low percentage of this type in this series. In a total of 69 cases of lobar pneumonia 5.7% had type 1 pneumococcus in the throat; 17.2%, type 2 (including atypical); 2.9%, type 3; and 60.8%, group 4. No pneumococci could be demonstrated in 13.0% of the cases. Of a total of 34 cases of bronchopneumonia, 5.8% showed the presence of type 1 pneumococci, 8.8% type 2, 2.9% type 3, and 52.6% group 4. No pneumococci were found in 29.4% of these cases.

It will be noted, from table 1, that about 60% of the cases of other diseases showed the presence of pneumococci in the throats. About 80% of the types, however, belong to group 4. Type 1 was not found in any case. Type 3, which occurs in normal throats to a certain extent, was present in 2.1% of the cases. No pneumococci could be demonstrated in 41.6%.

Of these 48 cases of other diseases, four of the throat cultures showed the presence of type 2 pneumococcus in the plate cultures. In one case of diabetes, types 4 and 2 were demonstrated on the plate cultures, only type 4 being recovered from the mouse. In a case of pyelocystitis, pneumococci of the sub 2 group were recovered from the mouse 8 days after injection. A type 2 organism was isolated from the throat of a case of spastic paraplegia and mental deficiency. The presence of this type of pneumococcus in the throats of these children,

not ill with pneumonia, may be due to contact. Nobécourt[12] and others have shown that the pneumococcus is readily transferred in children, and that a carrier of type 2 pneumococcus spreads the organism to other patients in the same hospital room.

The group of nonrespiratory diseases studied included the following: Gastro-enteritis, diabetes, colitis, pericarditis, diphtheria, acute lymphatic leukemia, dyspepsia, encephalitis, acute myocarditis, chronic empyema, congenital heart disease, spastic paraplegia, endocarditis, nephritis, mal-

TABLE 1

INCIDENCE OF PNEUMOCOCCUS TYPES IN CHILDREN

	Lobar Pneumonia					
	Uncomplicated (59 Cases)		Complicated (10 Cases)		Total (69 Cases)	
	Total Incidence %	Calculated Pneumococcus Incidence %	Total Incidence %	Calculated Pneumococcus Incidence %	Total Incidence %	Calculated Pneumococcus Incidence %
Type 1	6.7	7.6	0.0	0.0	5.7	6.5
Type 2	16.9	19.1	20.0	25.0	17.2	19.7
Type 3	1.6	1.8	10.0	12.0	2.9	3.3
Group 4	62.7	71.1	50.0	62.5	60.8	69.8
No pneumococci	11.8	....	20.0	....	13.0	....
	Bronchopneumonia					
	Uncomplicated (23 Cases)		Complicated (11 Cases)		Total (34 Cases)	
Type 1	8.7	12.5	0.0	0.0	5.8	8.3
Type 2	8.7	12.5	9.9	13.4	8.8	12.5
Type 3	4.3	6.2	0.0	0.0	2.9	4.1
Group 4	47.8	68.7	63.6	86.5	52.6	75.1
No pneumococci	30.4	....	27.2	....	29.4	....

	Other Respiratory Diseases (13 Cases)		Non-Respiratory Diseases (48 Cases)	
	Total Incidence %	Calculated Pneumococcus Incidence %	Total Incidence %	Calculated Pneumococcus Incidence %
Type 1	0.0	0.0	0.0	0.0
Type 2	30.7	33.3	8.3	14.2
Type 3	0.0	0.0	2.1	3.6
Group 4	61.5	66.6	47.9	82.1
No pneumococci	7.6	....	41.6	....

nutrition, cardiac decompensation, measles, otitis media, chronic appendicitis, diarrhea, surgical cases, typhoid, pyelitis, scarlet fever.

SUMMARY

In a series of 69 cases of lobar pneumonia and 34 cases of bronchopneumonia occurring in children between the ages of 3 weeks and 11 years, pneumococci were obtained by nasopharyngeal swabbing in 60.8% of the cases of lobar pneumonia and in 52.6% of the cases of bronchopneumonia.

[12] Presse méd., 1920, 28, p. 313.

The relative incidence of pneumococcus types in these two groups of cases was much the same, group 4 predominating, type 2 coming next in frequency, type 1, third and type 3 being least frequent. The fixed types of pneumococcus are less frequent in the pneumonias of children than in those of adults. The association of group 4 with representatives of one or another of the fixed types was demonstrated in several instances.

In a group of 13 cases of non-pneumonic respiratory disease in children, pneumococci were present in 92.0%. No examples of types 1 and 3 were encountered, type 2 and group 4 forming ⅓ and ⅔ respectively of the isolated pneumococci.

In 48 nonrespiratory diseases in children, pneumococci were found in 58.0%. Type 1 was not represented in this group, type 2 formed 14.2%, type 3, 3.6%, and group 4, 82.1%. The occurrence of type 2 pneumococci in the throats of these children in the hospital wards may have been due to contact.

REMOVAL OF ACIDFASTNESS FROM TUBERCLE BACILLI BY OLEIC ACID OR OLIVE OIL

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Since it was first observed by the author [1] that oleic acid or olive oil removes the acidfast property of tubercle bacilli when brought into contact with the dehydrated cultures, various conditions required for a successful reaction other than those described in these papers have been established. It is the purpose of this paper to tabulate additional factors that influence the removal of acidfastness from the bacilli. The H37 strain (Saranac Laboratory) which is a human type of tubercle bacillus has been used for the experiments. The first observation of the author was that the cultures after rapid dehydration with alcohol loose their acidfastness, become granular and undergo more or less disintegration when brought into contact with oleic acid at incubator temperature (table 1). It was first thought that oleic acid might contain a lipase that produced the effect, but no evidence was obtained to support this idea. The loss of acidfastness made its appearance after one hour at 37 C. and by the end of 24 hours there were only 1 to 10 acidfast bacilli in oil-immersion fields that contained several thousand blue-staining (Ziehl-Neelson) bacilli. A trace of water was found to be necessary for a successful reaction. Olive oil instead of oleic acid was added to the cultures dehydrated with 95% alcohol without effect on the bacilli. However, incubation with olive oil after dehydration with acetone gave results similar to those obtained by using the oleic acid on the alcohol-dehydrated bacilli. It was found that acetone could be used instead of alcohol for rendering the cultures non-acidfast by means of oleic acid. Some effect was obtained by incubating the acetone-dehydrated cultures with paraffin oil (liquid petroleum) but usually comparatively few bacilli were rendered non-acidfast by incubation with several samples of pure liquid petrolatum after the usual acetone dehydration.

From these results, set forth in earlier papers, arose the envelope idea that it is necessary to bring the oleic acid or olive oil into immediate contact with the bacilli by dehydrating with an agent in which the oleic acid or olive oil is soluble in order to permit the formation of an aqueous envelope immediately about the bacilli upon shaking the bacilli bathed

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[1] Amer. Rev. Tuberc. 1923, 8, p. 393; 1924, 9, p. 464.

TABLE 1

EFFECT OF OLEIC ACID ON ACIDFASTNESS OF TUBERCLE BACILLI

Dehydration	Medium	Results, in Approximate Percentages, of Non-acidfastness
50 cc. of 95% ethyl alcohol for 1 minute	10 cc. oleic acid plus 1 drop water for 48 hours at 37 C.	99
50 cc. absolute alcohol for 1 minute (3 changes)	2 cc. oleic acid for 24 hours.....	0
	On addition of 1 drop of water, and a second incubation of 24 hours	50
50 cc. of 95% ethyl alcohol for 1 minute and; (a) culture autoclaved at 20 lbs. pressure for 15 minutes; (b) dry in dry-heat oven at 100 to 150 for 4 hours	10 cc. oleic acid plus 1 drop of water for 48 hours at 37 C.	0
50 cc. of 95% ethyl alcohol for 1 minute	(a) 10 cc. oleic acid plus 5 cc. water for 48 hours at 37 C.; (b) 2 cc. absolute alcohol plus 2 drops of oleic acid; (c) 10 cc. water plus 0.5 gm. castor bean lipase (Tanaka)	0
50 cc. acetone for 1 minute.....	10 cc. oleic acid plus 1 drop of water for 48 hours at 37 C.	99
50 cc. of 95% ethyl alcohol for 1 minute	10 cc. oleic acid plus 1 drop of water at 37 C. for 1 hour	0
	4 hours.....	10
	12 hours.....	50
	24 hours.....	99

The usual method of dehydration is to decant the bouillon completely and cover the growth with the dehydrating agent which is quickly and completely decanted.

The oleic acid or olive oil is vigorously shaken with the water or other ingredient at the time of using.

TABLE 2

EFFECT OF OLIVE OIL ON ACIDFASTNESS OF TUBERCLE BACILLI

Dehydration	Medium	Results, in Approximate Percentages, of Non-acidfastness
10 cc. acetone for 5 minutes, after sedimenting by addition of a volume of acetone equal to that of bouillon	10 cc. olive oil plus 1 drop of water for 48 hours at 37 C.....	99
	24 hours at 2-10 C.....	0
	25 C.....	0
	30 C.....	15
	34 C.....	99
	48-50 C.....	75
	55 C.....	0
	10 cc. olive oil plus 2 cc. of water for 24 hours at 37 C.....	10
	10 cc. olive oil, heated in autoclave at 20 lbs. for 15 minutes, then plus 1 drop of water.....	99
Same treatment after washing growth with 2 changes of 10 cc. salt solution	10 cc. olive oil plus 1 drop of water for 48 hours at 27 C.....	99
50 cc. acetone in each of 3 changes during 15 minutes	10 cc. olive oil without water.....	0
10 cc. ethyl alcohol, 95%, after sedimenting by addition of a volume of alcohol equal to that of bouillon	10 cc. olive oil plus 1 drop of water for 48 hours at 37 C.....	0

in the dehydrating agent with olive oil or oleic acid in which small droplets of water are suspended. The individual bacilli or clumps of bacilli become encapsulated in thin aqueous envelopes and are sealed in oily compartments.

The further investigation of the nature of the loss of acidfastness which is presented in the present communication dealt first with an accurate determination of the temperature range required for the reaction (table 2). The failure of the reaction at 55 C. and at 25 C. makes it quite evident that the loss of acidfastness is not accomplished through a simple chemical process. It is especially significant that the change in the bacilli ceases between 50 and 55 C. which is a temperature that endangers the life of the tubercle bacillus. At 30 C. comparatively few of the bacilli become completely non-acidfast but a great many of the acidfast ones exhibit irregular swellings especially at the ends of the rods, a granulation, and an actual disintegration into acidfast granules. These changes in morphology always precede and accompany the loss of acidfastness. The reaction takes place best at temperatures at which the metabolic and growth activities of the bacilli are greatest, but extends definitely both below and above these optimum temperatures. The time required for the reaction has been determined (table 1). There is a latent period of at least one hour and little change takes place during the first four hours. After this time the loss of acidfastness progresses until almost all of the bacilli color blue by the Ziehl-Neelson method at the end of 24 hours of incubation. Always, however, a few acidfast rods persist, and it seems that they do persist because they are dead when placed in the incubator.[1] The required solubility of the oily medium in the dehydrating agent and the amount of water suspended in the oil which is necessary for the reaction are indicated in tables 1 and 2.

The conditions under which the loss of acidfastness takes place are not those imposed by a simple chemical reaction. That the dehydrated bacilli remove the water from its temporary suspension in the oily medium is certain and that they become intimately surrounded by an aqueous envelope seems likely. Within this capsule of water the living bacilli undergo changes which resemble microscopically the bacteriolytic changes seen in non-acidfast bacilli, and the necrobiotic changes of animal cells. Since only living bacilli are affected the loss of acidfastness is dependent upon some activity of the bacilli after they are encased in the oily medium and placed in the incubator. The ultimate explanation is uncertain. The traces of water about the culture may merely serve as a medium for the action of lytic substances excreted by the bacilli and

confined to the aqueous capsule about them. However, it has not been shown in these experiments that a reaction between metabolic products and oily medium may not take place which in turn has a lytic action on the bacilli and causes them to loose their acidfast property.

SUMMARY

Cultures of tubercle bacilli dehydrated with acetone or alcohol loose their acidfastness upon incubation with oleic acid. Cultures dehydrated with acetone loose their acidfastness upon incubation with olive oil, but those dehydrated with alcohol do not. The loss of acidfastness is incomplete in either case with less than 1% of the bacilli retaining their property of staining red by the Ziehl-Neelson method. The few bacilli that remain acidfast are thought to be dead at the time of incubation.

Traces of water are necessary for the discharge of acidfastness. The water adheres to the dehydrated cultures in the oily medium.

The temperature at which the loss of acidfastness takes place most rapidly is 37 C. with an abrupt cessation at a lower temperature where the metabolic activities of the bacilli are greatly reduced and at temperatures above 37 C. which approach the thermal deathpoint of the cultures. The variations in the reaction with changes in temperature are not those to be expected were the process a simple chemical one.

TUBERCULOUS INFECTION IN GUINEA-PIGS TREATED WITH TUBERCLE BACILLI MADE NON-ACIDFAST BY INCUBATION WITH OLIVE OIL

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Dehydrated cultures of tubercle bacilli suspended in olive oil promptly loose their acid-fastness upon incubation, and the subcutaneous or intraperitoneal injection of these non-acidfast cultures into guinea-pigs sensitizes to the intracutaneous administration of old tuberculin.[1] The purpose of this paper is to present a series of results obtained by treating guinea-pigs with the non-acidfast cultures before and after inoculating them with living tubercle bacilli.

PREPARATION OF THE SUSPENSIONS OF NON-ACIDFAST BACILLI

The method of preparing the suspensions is as follows: The two glycerin broth cultures in 125 cc. Erlenmeyer flasks with the growth covering the entire surface of the medium are combined, and all but 10 cc. of the broth removed with a bulb pipette. Under the best conditions the film of the growth will spread over the entire broth surface during two weeks of incubation. To the culture and remaining broth, 100 cc. of pure acetone are added. The clear acetone is decanted after one minute and the growth is again covered with 100 cc. of acetone. After a contact of two minutes this also is decanted. Immediately the dehydrated culture is covered with 10 cc. of pure olive oil that has been shaken vigorously with one drop of distilled water. After the culture and oil have been mixed by rotating the flask the mixture is placed in the incubator at 37 C. for 24 hours. Smears are made on slides and the oil removed from them by carefully dropping xylol on the slides. The smears are then fixed in the flame and stained by the Ziehl-Neelsen method. There are about 5 to 10 acidfast bacilli per oil immersion field with the great mass of bacilli staining blue. There are several variable factors that influence the dehydration and it appears to be due to these especially that the loss of acidfastness in some instances does not take place as it should. The culture is discarded if the loss of acidfastness is not satisfactory. To remove the oil after incubation 100 cc. of acetone are added to the non-acidfast culture, and after 3 minutes the suspension is poured on a paper filter. The precipitate is washed with 100 cc. of acetone and at once suspended in 40 cc. of salt solution. The suspension is made by rubbing the oil-free precipitate in a mortar with a few drops of saline solution and finally adding the entire 40 cc. The suspension is heated in the Arnold sterilizer for 30 minutes.

Two cultures of the human type have been employed. The H37 strain (Saranac Laboratory), although of relatively low virulence produces a progressive tuberculosis in guinea-pigs. The H60 culture (Saranac Laboratory) is much more virulent and causes a fatal infection in guinea-pigs in eight to twelve weeks. The H37 culture is made non-acidfast with greater ease.

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[1] Am. Rev. Tuberc., 1924, 9, p. 464.

GUINEA-PIGS TREATED WITH NON-ACID FAST SUSPENSIONS AND INOCULATED WITH GROWING ORGANISMS OF THE SAME VIRULENT STRAIN (TABLE 1)

The animals were inoculated with living cultures on May 25, 1925. Suspensions for inoculation were prepared by rubbing a mm. mass of a rapidly growing culture of tubercle bacillus (H60) in a mortar with a few drops of 10% gelatin solution, and finally adding salt solution, to 25 cc. After centrifugation, the supernatant fluid showed 1 to 5 acidfast bacilli per oil-immersion field when fixed to a slide by allowing a small drop to dry. One cc. of this was added to 100 cc. of salt solution. Injection with the non-acidfast suspensions were made before or after the inoculation. The animals alive on the 70th or 140th day were chloroformed. At necropsy, all animals were tuberculous. The two control guinea-pigs that lived 70 days showed a greater number of tuberculous foci in the internal organs and a less tendency to form fibrous tissue and giant cells than the larger and older guinea-pigs that were given suspension injections. The tuberculosis in four guinea-pigs treated with non-acidfast suspension after inoculation also appeared to be somewhat less than in the controls, and a second experiment was made to determine in animals of more nearly the same weight whether treatment with the bacillus suspensions had any effect on the development of the disease. Thirty-three guinea-pigs were inoculated intraperitoneally on October 27. Of these, 15 subsequently received at ten-day intervals 6 doses of 0.1 cc. of non-acidfast suspension. Twelve were given at ten-day intervals 6 doses of 0.1 cc. of old tuberculin, while the six of the inoculated animals received no further treatment. On the 70th day or the 90th day following inoculation the animals were chloroformed.

There was no substantial difference between those treated with suspensions of the non-acidfast bacilli, those receiving old tuberculin, and the controls.

EXPERIMENTS WITH A LESS VIRULENT CULTURE OF TUBERCLE BACILLUS

Instead of the suspension of the non-acidfast bacilli in normal saline solution the mixtures of dehydrated bacilli and olive oil were kept at 37 C. for four or five days and these oil mixtures injected. Injections of the bacillus-oil mixtures were followed by much reaction with local induration which often persisted for several weeks. More than 100 guinea-pigs were injected with the oil-bacillus suspensions and subsequently inoculated with the same strain. The animals were divided into a number of groups with variations in the size of the doses and the length of time elapsing between suspension injections and inoculations.

Of these animals a few small groups illustrate the results. In group 1 of 9 animals 6 were injected subcutaneously with 4 cc. of the oil suspension (a very large dose) in eight places, and 22 days later these animals with one exception together with 3 normal untreated ones were inoculated with a large dose of tubercle bacilli (1 cc. of a suspension showing in smears 1000 bacilli per oil-immersion field). At necropsy, at the end of 5 months there was no difference between the treated and control animals. The one given suspension but not

inoculated showed no caseation in the groin and no tubercles in the internal organs. Group 2 consisted of 18 animals. Of these 14 were injected with 2 cc. of the oil suspension of H37 and 39 days later inoculated together with 4 controls with virulent bovine bacilli (1 cc. of a suspension showing about 1 bacillus per oil-immersion field). At necropsy, 55 days later all animals showed an extensive tuberculosis with no difference between those injected with suspension and the controls. In group 3, a longer time elapsed between treatment and inoculation. The first dose was 2 cc. of the oil suspension which was repeated 7 days later. The inoculations were made 3 months later with 0.2 cc. of a suspension of the same strain (5 bacilli per oil-immersion field). These animals were chloroformed 7 months after inoculation. In all control animals there were numerous tubercles in the spleen and fewer in the liver and lungs. In several of the guinea-pigs tubercles were not seen in the gross in the spleen but microscopically small tubercles were found. There was no pronounced difference between the 17 treated and the 4 untreated guinea-pigs, but as a group the tuberculosis appeared to be somewhat more extensive in the control animals that received no suspension.

DISCUSSION

A prevalent view is that there exists a parallelism between the immunity (resistance to reinoculation) of a tuberculous animal and the response to the intracutaneous administration of tuberculin. In the non-tuberculous guinea-pigs (table 1) that react to intracutaneous tuberculin following the treatments with non-acidfast suspensions an increased resistance to tuberculosis is difficult to demonstrate, if it exists. Many intracutaneous tuberculin tests were made on the animals before inoculation with living tubercle bacilli. Frequently the tests resulted in small necrotic foci, but more often the response consisted only of an area of induration and hyperemia. One guinea-pig (non-tuberculous) was given the usual intracutaneous test May 27, 1925, which was 33 days after the intraperitoneal injection of 2 cc. of the non-acidfast suspension in saline solution. At the end of 48 hours, the reaction consisted of a 3 cm. red indurated area with a 5 mm. necrotic center. At the end of 6 days, there was still local induration at the site of the test. On July 6, the test was repeated with the same intensity of reaction. In guinea-pig 581 which received subcutaneously 0.2 cc. of the same suspension the reaction to intracutaneous tuberculin at the end of 48 hours consisted of a 1 cm. red indurated area without necrosis. Briefly the average reaction to tuberculin obtained in testing

TABLE 1

EFFECT OF TREATMENT OF GUINEA-PIGS WITH NON-ACIDFAST TUBERCLE BACILLI

Number	Weight in Gm. at Time of First Treatment or Inoculation	Treatment with Oil-free Suspension of Non-acidfast Tubercle Bacilli	Inoculation with Culture Suspended in Gelatin and Salt Solution, May 25, 1925	Result
1	552	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	1 cc. subcutaneously in right groin	Aug. 4, 1925, chloroformed. Ulcer groin; tubercles in spleen raised above surface. Microscopically many tubercles with fibrous tissue, giant cells, and lymphocytes
2	679	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	1 cc. subcutaneously in right groin	August 4, chloroformed. Similar to 1
3	623	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	1 cc. subcutaneously in right groin	August 4, chloroformed. Similar to 1
4	495	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	Not inoculated	Died, April 27
5	624	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	Inoculated	August 4, chloroformed. Similar to 1
6	652	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	Not inoculated	Died, April 25
7	567	Apr. 24, 1925, 2 cc. intraperitoneally; May 6 and 19, 1 cc. subcutaneously	Inoculated	August 4, chloroformed. Similar to 1
8	637	Apr. 24, 2 cc. intraperitoneally	1 cc. subcutaneously in right groin	August 4, chloroformed. 5 mm. ulcer in groin. Gross and miscroscopic lesions like 1
9	630	Apr. 24, 2 cc. intraperitoneally	1 cc. subcutaneously in right groin	August 4, chloroformed. Similar to 8
10	550	Apr. 24, 2 cc. intraperitoneally	1 cc. subcutaneously in right groin	August 4, chloroformed. Similar to 8
11	353	June 1, 15 and 29; July 7 and 20, 1925 0.1 cc. subcutaneously	1 cc. subcutaneously in rigbt groin	Sept. 17, 1925, chloroformed. Weight 760 gm. Lymph node in groin adherent to ulcerated skin. Spleen and liver not enlarged. Microscopically tubercles with fibrous tissue, giant cells and lymphocytes
12	375	.....	1 cc.	Aug. 4, 1925, dead. Huge liver and spleen with 2 cm. caseous areas not elevated above surface of organ. Microscopically absence of fibrous tissue, giant cells and lymphocytes in the tubercles
13	360	.....	1 cc.	June 3, dead. Extensive tuberculosis
14	380	.....	1 cc.	July 18, dead. Huge liver and spleen
15	390	.....	1 cc.	August 4, chloroformed. Similar to 12

a great number of guinea-pigs injected with the non-acidfast bacilli has been distinctly less pronounced than the usual reaction seen in the tuberculous guinea-pigs. Although most of the animals were inoculated when their nutrition was good and when the tuberculin test was positive, no increased resistance to the infection could be demonstrated regularly in an entire series of animals. This was especially true in the case of the virulent strain. The fact that the resistance of the guinea-pig is not increased to a degree that arrests the progress of the infection is not proof that injections of the non-acidfast suspensions might not influence favorably the course of tuberculosis in animals with greater natural immunity to the disease, but on the other hand, no evidence has been presented in these experiments which indicates that such is the case.

Petroff [2] obtained in guinea-pigs injected with heat-killed tubercle bacilli a positive intracutaneous reaction with tuberculin. Yet it is difficult to demonstrate in guinea-pigs treated with dead bacilli any distinct resistance to tuberculous infection.[3]

In a small series of guinea-pigs injections of tuberculin (O. T. Mulford) appeared not to influence the progress of the infection. Corper [4] found that the administration of old tuberculin had little or no effect on the course of tuberculosis in the guinea-pig.

SUMMARY

Tuberculous infection of guinea-pigs produced by inoculation with virulent tubercle bacilli (Saranac Laboratory H60) is not influenced by injections of saline suspensions of cultures of the same strain of bacilli made non-acidfast by dehydration and incubation with olive oil. The results are the same whether the injection of non-acidfast suspensions precedes or follows the inoculation.

Mixtures of non-acidfast tubercle bacilli of a lesser virulence (Saranac Laboratory H37) and the olive oil in which they were incubated were injected into several series of guinea-pigs before inoculation with living cultures of the same strain of organism. The animals treated with the oil-bacillus suspensions became tuberculous upon incubation and there was no material difference in the progress of the infection in the treated guinea-pigs and untreated ones of the same age and weight.

[2] J. Immunol., 1924, 9, p. 309.

[3] Smith, Theobald: Harvey Lecture, Parasitism of the Tubercle Bacillus and Its Relation to Infection and Immunity, 1906, p. 272.

[4] Am. Rev. Tuberc., 1918, 2, p. 600.

A SKIN REACTION IN POLIOMYELITIS

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The need for a susceptibility test in poliomyelitis has become especially apparent through the results obtained by the precipitin test with cleared extracts of nasopharyngeal swabbings and the poliomyelitis antistreptococcus serums.[1]

My repeated attempts to obtain a suitable antigen to test susceptibility were unsuccessful until last summer, when it was found that freshly isolated strains of the pleomorphic streptococcus, which produced flaccid paralysis in rabbits, when grown 18 to 24 hours in pancreatic digest heart muscle broth to which one part in ten of ascites fluid was added, and the culture killed with phenol, 0.5%, or tricresol, 0.3%, yielded a useful toxic antigen. Tunnicliff [2] has since reported on the use of a similarly prepared antigen with the diplococcus which she isolated in cases of measles. One-tenth cc. of a 1 : 100 dilution of this killed culture in sodium chlorid solution, injected intradermally, resulted in no reaction or only slight redness in persons who had recovered from poliomyelitis, but redness was often marked if persons had not had poliomyelitis. The maximal reaction develops in from 18 to 24 hours, after which it gradually subsides. Slight induration and redness limited to the area injected may last for several days. Absence of redness at the point of injection of the test substance at the end of 18 to 24 hours is considered negative; redness limited to the area injected is recorded as +; redness extending beyond the blistered area approximately 1 cm. in diameter, as + +; redness from 1 to 2 cm. in diameter as + + +, and larger areas usually from 2 to 3 cm. (more rarely 4 cm. in diameter) as + + + +. The reactions of + and + + are considered as mildly positive and those of + + + and + + + + as strongly positive. The control injection consists of a dilution of 1 : 100 of the corresponding uninoculated broth. This has always been negative, or there has been only a slight redness limited to the area blistered at the time of the injection. The results obtained thus far are summarized in the accompanying tabulation.

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[1] Rosenow, E. C.: J. Am. M. A., 1925, 84, p. 429.
[2] J. Infect. Dis., 1925, 37, p. 193.

Strongly positive reactions were not obtained in persons who had recovered from typical or abortive attacks of poliomyelitis, from three weeks to many years previously. In all of four cases thus far tested during the acute stage of the disease the reaction was strongly positive. In two of these the reaction was negative, 3 and 7 weeks later, respectively. In one the reaction was strongly positive on the 3rd day after onset of the disease, mildly positive on the 8th day, and negative on the 21st day. As noted in the last column of the tabulation, the percentage incidence of a strongly positive skin reaction corresponds fairly well with the age incidence in poliomyelitis. The incidence, including all ages, in family groups was 41%; in children up to five years of age, 67%;

TABLE 1

RESULTS OF A SKIN TEST IN POLIOMYELITIS

Groups and Ages	Number	Reactions			
		Negative	Mildly Positive	Strongly Positive	
				Number	%
Persons who have recovered from typical poliomyelitis: all ages	41	30	11	0	0
Persons who have recovered from abortive poliomyelitis: all ages	12	9	3	0	0
Normal persons in family groups: all ages	60	17	18	25	41
Normal persons: 1 to 5 years	9	0	3	6	67
Normal persons: 6 to 10 years	45	5	7	33	73
Normal persons: 11 to 15 years	31	0	17	14	45
Normal persons: 14 to 19 years	98	19	52	27	28
Normal persons: 16 years and older	136	67	48	21	15
Total: all ages	379	108	145	126	33

from six to ten years, 73%; from 11 to 15 years, 45%; from 14 to 19 years, 28%; and in adult persons 16 years or more, 15%. Of a total of 379 persons of all ages who have not had poliomyelitis, the reaction was strongly positive in 33%. Dr. E. T. Evans of LaCrosse, Wisconsin, applied the skin test at an orphanage to 141 children ranging from 2 to 17 years of age, about two months after the occurrence of three cases. The reaction was strongly positive in 89 (63%). I tested 85 children of the same age group and the reaction was strongly positive in 53 (62%). In Dr. Evans' group, as in mine, a slightly lower incidence of a strongly positive reaction was obtained in persons who reacted positively to the precipitin test, and who had remained free from symptoms during the epidemic, but such persons who had frank, or abortive attacks of poliomyelitis at the time or subsequently, and who gave a positive precipitin reaction, reacted negatively or only slightly positively to the skin test in each instance. In some instances, persons with marked

residual paralysis have reacted negatively to undiluted killed cultures to which those highly susceptible reacted positively in dilutions as high as 1 : 1000. The reaction appears fairly specific. The incidence of positive reactions with similar antigens, prepared from other strains of green producing streptococci or pneumococci and the Dick toxin, was about as high in persons that had recovered from poliomyelitis as in normal controls. The skin of most rabbits has been found susceptible, while the skin of the Macacus rhesus and Java monkeys and the baboon, has been found insusceptible; results in agreement with the difference in susceptibility of these animals to inoculations of the streptococcus.

Seven different strains isolated a short time previously were found satisfactory while several old strains were unsatisfactory. The diluted suspensions tend to deteriorate, and should not be used later than ten days after dilution. The undiluted killed cultures also tend to become unsatisfactory in from one to three months. In several instances marked increase in nonspecific toxicity occurred after prolonged preservation of the killed culture in the ice chest. Filtrates of broth cultures have thus far been found nontoxic.

SUMMARY

The absence of marked reactions in persons fully recovered from poliomyelitis and who are known to be immune; the incidence of positive reactions inversely according to age, corresponding in general to the age incidence of poliomyelitis; the strongly positive reactions during the acute stage of the disease, and the negative reaction during convalescence, are considered as presumptive evidence that the test is a measure of susceptibility to poliomyelitis.

Numerous questions regarding the nature of the reaction have not yet been worked out. However, the immune serum prepared from horses with the pleomorphic streptococcus, and used with apparent benefit in the treatment of the early stages of poliomyelitis, has a marked neutralizing power over the toxin, as determined by the skin reaction.

FURTHER STUDIES OF THE POLIOMYELITIS PRECIPITIN REACTION

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By inoculating rabbits intracerebrally, with suspensions of nasopharyngeal washings, I have demonstrated the prevalence of the pleomorphic streptococcus believed to be of etiologic significance in poliomyelitis, in the throats not only of persons ill with typical and abortive attacks, but of normal persons during epidemics of poliomyelitis. By the same method this organism was proved absent in the throats of these subjects during the absence of poliomyelitis. In control experiments performed during epidemics of the last two summers it was found that, when animal inoculations revealed the presence of this organism, the cleared salt-solution extracts of nasopharyngeal swabbings usually gave a positive precipitin reaction with the hyperimmune horse serum, which is prepared by injection of the pleomorphic streptococcus, and used in the treatment of poliomyelitis.

With the precipitin test the presence of this streptococcus was demonstrated during the epidemic in Detroit in 1924,[1] not only in the throats of patients with poliomyelitis (83%), but also in the throats of normal contacts (77.5%), of patients with various diseases (65%), of patients with scarlet fever (24%), and of normal persons within the epidemic zone (59%), whereas a uniformly low incidence was revealed in three control groups outside the epidemic zone: patients with various diseases (4.8%), patients with scarlet fever (9.5%), and normal persons (5.5%). Further studies with the precipitin reaction during the last summer, made in connection with three epidemic outbreaks, corroborate fully the results obtained last year, and have extended our knowledge of the relationship of the streptococcus to poliomyelitis in important respects. It is my purpose in this paper to report the newer findings.

Technic.—The technic was essentially the same as that used in 1924.[1] It was made as uniform as possible throughout the study. The swabbings were made from the nasopharynx in the same manner, gross contamination from the tongue being avoided. I made readings under the same conditions of illumination in a darkened room, and in order not to be biased in recording findings, I often made

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[1] Rosenow, E. C.: J. Am. M. A., 1925, 84, p. 429.

them without knowing at the time, the source of the extracts. In many instances tests on duplicate swabbings were made at the same time and repeat swabbings at short intervals. At least two different preparations of the poliomyelitis antistreptococcus serums and four control serums were used throughout the study. In some instances the precipitating power of the serum of convalescent human beings and monkeys was also tested. Blood agar platings of suspensions of the swabbings were made in many instances, both during and following the epidemic.

The results in patients with the frank and abortive forms of poliomyelitis; in contacts, consisting usually of the other members of a household with one case of the disease; and in controls, persons not exposed, within and without the epidemic zone, are summarized in table 1. Only those patients and contacts are included who had swabbings within ten days of the onset of symptoms. The high incidence of positive reactions (97% in the 77 frank cases of acute poliomyelitis, 94% in 109 cases of abortive poliomyelitis, and so forth) represents, with few exceptions, the result of a single swabbing in each case. The incidence of positive

TABLE 1

PRECIPITIN REACTION WITH EXTRACTS OF NASOPHARYNGEAL SWABBINGS AND POLIOMYELITIS ANTISTREPTOCOCCUS SERUM

Groups	Number of Persons	Positive Reactions	
		Number	%
Patients with acute poliomyelitis	77	75	97
Patients with abortive poliomyelitis	109	103	94
Contacts	319	240	75
Controls (within epidemic zone)	777	562	72
Controls (outside epidemic zone)	264	44	17

reactions in the contact (75%) and control groups within the epidemic zone (72%) was much higher than in a similar control group outside the epidemic zone (17%). Ninety-four swabbings representing 77 cases were made from the second to the tenth day, inclusive, after onset of symptoms. Of these 88 yielded positive, and six, negative reactions. Sixteen swabbings in 14 cases were made from the 11th to the 21st day. Of these, 9 were positive and 7 negative. Twenty-one swabbings were made, from 22 days to 18 weeks after the attack, from 21 patients residing outside the epidemic zone. Of these, only two reacted positively with the poliomyelitis serums. Seven patients with nasopharyngitis, 8 with whooping cough, 4 with chicken pox, 7 with acute follicular hemolytic streptococcus tonsillitis, 1 with erysipelas, 3 with scarlet fever, and 5 with scarlatinal hemolytic streptococcus infection of the throat, served as additional controls. The swabbings in all these cases reacted negatively with the poliomyelitis serums, but the swabbings in

the cases of scarlet fever and scarlatinal infection of the throat without rash reacted positively with my old scarlatinal immune serum and with the Dick scarlatinal antitoxin.

Last summer I had the opportunity to study the precipitin reaction during and following three community and two institutional outbreaks of poliomyelitis. The first epidemic, comprising 14 cases, occurred in and around Galesville, Wisconsin, a town of 1,000 inhabitants; the second, including 11 cases, occurred in the township of Waumandee, Wisconsin, and surrounding country, and the third, comprising 22 cases, occurred in and around Rochester, Minnesota. The reactions in the community and institutional outbreaks, together with control swabbings made within 24 hours after the patient's arrival in Rochester, are summarized in table 2. The incidence of positive reactions during the epidemics at Galesville (62%) and Waumandee (80%) was much higher than that in control swabbings made at the same time (14%) in Rochester, before the epidemic appeared there. By means of these control swabbings it was determined that the incidence of positive reactions in Rochester became high with the appearance of the epidemic and declined as the epidemic subsided. Thus, during the three weeks preceding the occurrence of the first case only 14% of the swabbings from 102 persons reacted positively to the precipitin test, whereas during the epidemic period of six weeks, 70% of the swabbings from 632 persons reacted positively, and again, during the four weeks following the occurrence of the last case, only 13% of the swabbings from 265 persons gave a positive reaction.

The results at the two orphanages (Wabasha, Minnesota, and La Crosse, Wisconsin) were similar. Dr. E. T. Evans made the swabbings at the latter institution. The incidence of positive reactions was high in both places at the time the cases occurred (57 and 49%), and low (8 and 10%) three months and six weeks, respectively, afterward. Swabbings of patients at the Mayo Clinic, representing many states, taken August 26 and 27 when poliomyelitis was generally prevalent, yielded positive reactions in 33% of 290 cases, while on October 29 and November 2, after several weeks of cold weather and after poliomyelitis had largely disappeared, only 5% in 297 cases reacted positively. After the epidemic in Rochester had subsided and the precipitin tests had largely become negative, I had an opportunity to make precipitin tests in family groups in connection with ten scattered cases of poliomyelitis that occurred in the country from 30 to 90 miles south of Rochester. In the swabbings of all of the patients and of 52% of 29

contacts the reaction was positive, in contrast to only 6% of 36 controls outside the epidemic zone.

The precipitin reaction has diagnostic value chiefly in sporadic cases, and in cases that develop shortly before or during the early part of an epidemic, or as it subsides, but not during the height of an epidemic when too many normal persons have positive swabbings. The reaction was positive in not less than nine instances of children who were ill with nausea, vomiting, fever, and other symptoms that could not be explained as due to other causes, when the swabbings from the other children in the respective households who were well reacted negatively. The reactions were also the same in cases in which paralysis developed. Thus, at the outset of the epidemic in Rochester, all the swabbings made July 31 in a family of seven proved negative except that of the child who was ill, and who developed paralysis two days later. August 6 all the re-swabbings proved positive, except that of the mother. August 12 the reactions for the patient who had received serum treatment and for all but one of the children were negative. The child with the positive reaction had felt badly, and had had fever August 9 and 10. Swabbings made August 18 proved negative in all cases. The tests were repeated October 9, at which time all reactions were negative except in the case of the mother, whose swabbing had reacted negatively in each of the four previous tests.

The incidence of positive precipitin reactions among the parents and other adults in family groups during the epidemic period was found to be almost as high as that among children. Thus, for 194 parents and other adults, 125 reactions (64%) were positive, and for 259 children whose throats were swabbed at the same time, 190 reactions (73%) were positive. Four children and one adult developed typical attacks of poliomyelitis and 17 children and one adult developed abortive attacks within two weeks after the streptococcus had been demonstrated in the throat by means of the precipitin test. Only two children developed abortive attacks, and none of the persons for whom the precipitin reaction was negative developed typical poliomyelitis.

Of 11 persons who had had poliomyelitis in previous epidemics, the reactions for eight were positive and for three negative during the epidemic period. One of these was ill with fever, nausea and vomiting, and other symptoms resembling an abortive attack at the time the swabbing was done, and one developed a similar attack six days later. Both recovered without developing paralysis. In 8 of these 11 persons who had recovered from poliomyelitis during previous epidemics the throats

were re-swabbed several weeks after the epidemics of last summer had subsided. The reaction for only one was positive. Of 21 of the patients for whom reactions had been positive during the epidemic studied, only one had swabbings that reacted positively from three to twelve weeks after the epidemic had disappeared. Positive precipitin reactions were obtained during epidemics for persons varying in age from two months to 80 years. These include 15 (79%) of 19 infants, aged from 2 to 18 months. Six of these were contacts and 13 were controls within the epidemic zone.

Swabbings were taken August 3 from 114 persons residing in the city blocks surrounding two households at the time when there was a case of poliomyelitis in each. For 93 of these (81%) positive reactions were obtained. August 17, one week later, another case had developed in the same neighborhood, the throats of 78 persons were swabbed; the reactions for 62 (79.5%) were positive. In 35 of these cases, swabbings had been taken previously; in 24 (69%) the reaction was positive. In 21 cases the reactions were positive both times; in 8 they had become negative; in 3, they became positive, and in 3 the reactions were negative both times. One family, consisting of parents and three children, whose home was situated within a block of the three households in which the three cases had occurred, had been away on a vacation, returning the night of August 7. Swabbings taken the following morning proved negative in all cases. The children were kept in their own yard, away from other children in the neighborhood, the mother keeping close watch meanwhile. The father ventured out to his work at night, coming in contact with relatively few persons. August 13 re-swabbings were taken. All yielded positive precipitin reactions. The swabbings were repeated August 18. All of the three children had developed nausea, vomiting, or headache, with fever lasting for several days. The precipitin reaction proved negative for the father, mother, and the oldest daughter, and positive for the two younger children. Swabbings taken again, October 9, after poliomyelitis had disappeared, proved negative in all.

Similar results were obtained in other family groups. In three instances members of families had been separated during the epidemic period. For nearly all of those who had remained at home the reactions were positive to tests made just after those that had been outside the epidemic zone had returned home. For the latter the reactions were negative. Of seven children from three families that had been away and for whom the precipitin reactions immediately on their return were

negative, but soon afterward became positive, six developed febrile reactions and other symptoms resembling abortive attacks of poliomylitis within 12 days after their return. In other households for nearly all of those residing within the epidemic zone reactions were positive, when for friends who had arrived shortly before from places free from poliomyelitis, the reaction was negative. In sharp contrast to these were the findings in several families during the epidemic in Rochester. Swabbings were taken from the members of the household and from visiting friends from a neighboring city having cases of poliomyelitis. For both groups there was the usual high incidence of positive reactions. The incidence of positive precipitin reactions was high also for family groups in the country where no cases of paralysis developed, but mild illnesses sometimes resembling abortive attacks occurred in children at the time when poliomyelitis was prevalent in the community. The very close relation between the incidence of positive precipitin reactions and the incidence of cases of poliomyelitis was forcibly brought out in an experiment performed September 21, on swabbings taken one or two days before from 74 persons, of whom 59 resided in the township of Waumandee, Wisconsin, where the epidemic had disappeared several weeks before. For most of them the precipitin tests had been positive during the epidemic. Now in only three (5%) positive reactions were obtained. At one of these homes swabbings were also taken from a family of four, who resided in a valley seven miles away where several cases of poliomyelitis had occurred a short time previously and who were spending the day there. Their two children, twins three years of age, had undoubtedly had abortive attacks a week before. The reactions were positive for the whole family. The remaining 11 were members of two families who lived on farms 30 miles south of Rochester, where three cases of poliomyelitis had developed within the previous week. For seven (63%) of these there were positive reactions.

The rapidity with which this streptococcus may become prevalent in the throat is illustrated by the following. Swabbings were taken from 44 persons residing in Rochester, within 7 days before the first case occurred, and again within two weeks; meanwhile, five cases had developed. The reactions were positive for only two (5%) before poliomyelitis appeared, whereas they were positive for 32 (72%) after poliomyelitis had appeared. None of the 44 persons had been exposed, or related in any way to the families in which the cases occurred. Thirty-five patients who came to Rochester during the epidemic and who had registered at the clinic from four to ten days before had had

their throats swabbed August 25. The reactions were positive for 20 (57%) which is in sharp contrast to the incidence of 33% of 290 patients whose throats were swabbed August 26 and 27 (table 2), and who had arrived the day the swabbing was done, or just the day before.

The duration of the carrier state varies within wide limits, although usually it does not last longer than two or three weeks. It appears to persist longest in severe untreated cases of poliomyelitis, especially if the patient has large infected tonsils and adenoids. Of 55 persons in family groups who had had swabbing repeatedly, the reactions were positive to the precipitin test for 46 (83%) at the time when one member had poliomyelitis, whereas the reactions were positive for only eight (14.5%) about two weeks later.

TABLE 2

Precipitin Reaction in Relation to Epidemic Poliomyelitis

Community or Institution	Relation to Epidemic	Date, 1925	Number of Persons	Positive Reactions	
				Number	%
Galesville, Wis.; 14 cases	During	July 11, 21	233	146	62
	Following	November 9	79	4	5
Waumandee, Wis.; 11 cases	During	July 18, 21	77	62	80
	Following	September 20	60	4	7
Rochester, Minn.; 22 cases	Before	July 11 to 30	102	14	14
	During	August 2 to September 12	632	445	70
	Following	September 13 to October 10	265	34	13
Orphanage at Wabasha, Minn.; 3 cases	During	July 28	79	45	57
	Following	October 22	78	6	8
Orphanage at La Crosse, Wis.; 3 cases	During	September 12	144	71	49
	Following	October 30	144	14	10
Registrants at Mayo Clinic, Rochester, Minn.		August 26, 27	290	97	33
		October 29, November 2	297	14	5

It was hoped that the precipitin reaction with the spinal fluid in cases of acute poliomyelitis might prove of diagnostic value. However, only slightly positive reactions, visible only in strong illumination, were obtained in only 21 of 43 cases. Samples of spinal fluid obtained in other conditions for controls gave negative reactions in nearly every instance.

Attempts to prove parallelism between a positive precipitin reaction and the presence of poliomyelitis virus have thus far been unsuccessful, a finding in keeping with the fact that during epidemics of the last two years, contrary to those of former years, I have been unable to demonstrate virus in filtrates of nasopharyngeal washings of patients with acute poliomyelitis.

There are good reasons for believing that similar precipitin experiments with appropriate hyperimmune serums will prove of value in a study of other epidemic diseases in which infection of the upper respiratory tract occurs. In scarlet fever I have already demonstrated this.[2]

RESULTS WITH CONTROL SERUMS

A slight clouding at the point of contact of cleared nasopharyngeal extracts and normal horse serum occurred in only 31 instances (1.2%) of 2,442 tests made with this serum. The incidence of positive reactions with the type 3 pneumococcus serums, also used as controls, was approximately the same during and following the epidemics. With type 1 pneumococcus serum, 53 (5.2%) of 1,016 tests proved positive; with type 2, 55 (4.8%) of 1,132 tests, and with type 3, 40 (3.8%) of 1,043, tests proved positive.

Serum prepared by intravenous injection in a horse of non-scarlatinal hemolytic streptococci used as an additional control yielded positive results in only 17 (1.7%) of 972 tests, and the scarlatinal antistreptococcus serum yielded a positive reaction in 12 (5.6%) of 214 tests. Blood agar platings in these showed large numbers of hemolytic streptococci.

The bacterial flora of the throats in cases of poliomyelitis, as revealed by blood-agar plate cultures made as controls, are not characteristic, although the number of streptococcus colonies surrounded by a green zone is unusually large during the acute stage of the disease. Later, large numbers of slightly hemolytic streptococci are often found instead, but typical hemolytic streptococci are almost never encountered in this disease. The incidence of the hemolytic streptococcus on blood-agar plates was determined during and following the epidemic period. During the epidemic it was present in variable numbers in only 55 (6.7%) of 818 persons, whereas after the epidemic had subsided and throat infections and colds had become prevalent, it occurred in 89 (16.5%) of 538 persons.

SUMMARY

The results of the precipitin reaction with immune horse serums and extracts of nasopharyngeal swabbings in community and institutional outbreaks of poliomyelitis, proved positive in nearly all frank and abortive cases at the time of the attack, in a high percentage of normal contacts and in persons not exposed to the disease at the time when cases occurred. It proved negative in nearly all of the cases in from two to three weeks after the acute attack had subsided and in normal persons soon after the epidemic had disappeared. In one epidemic, the incidence of positive reactions generally was found low shortly before the occurrence of the first case, high during the period of the epidemic and again low after the epidemic had subsided. The increase in positive reactions as cases of poliomyelitis developed and the decrease as poliomyelitis disappeared, occurred rapidly and seemingly independently of exposure to the disease, in isolated households in the country as well as in the urban

[2] J. Infect. Dis., 1925, 36, p. 525; J. Am. M. A., 1926, 86, p. 9.

populations. Persons who were negative to the precipitin reaction on entrance into the epidemic zone soon became positive and reactions resembling abortive attacks of poliomyelitis were common in children. The number of positive reactions in persons who came to the Mayo Clinic from widely separated communities was relatively high during the latter part of August when poliomyelitis was generally prevalent and much lower during the latter part of October after poliomyelitis had largely disappeared. After the epidemic in Rochester had subsided and the precipitin reaction in the population had become largely negative, cases occurred south of Rochester where the number of a positive precipitin reaction was high.

In certain instances poliomyelitis occurred without exposure within from five to twelve days after the presence of the streptococcus was demonstrated in the throat. Repeated swabbings showed that the carrier state lasts usually from one to three weeks in normal persons. Immunity to poliomyelitis and the occurrence of the organism in the throat did not run parallel. The positive reactions during epidemics in adults, who are relatively immune, and in children, who are relatively susceptible, were found nearly equally high, and persons who had had poliomyelitis became carriers of the streptococcus during epidemics quite like persons who had not had the disease.

TULAREMIA IN WILD RATS

L. V. Dieter and Bernice Rhodes

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In the course of the routine examination of rats for plague infections following the outbreak of pneumonic plague at Los Angeles in the fall of 1924, occasionally guinea pigs inoculated with suspected tissues presented plague-like lesions. For convenience the findings may be divided into two classes. Those in which organisms morphologically resembling plague, but culturally not plague, could be demonstrated, and those in which no plague-like organisms were found even on repeated reinoculations.

The first class, which comprises several different organisms including B. myxoides and members of the paratyphoid-enteritidis group, exhibited marked virulence for guinea-pigs, even upon cutaneous inoculation. The gross anatomic lesions are somewhat similar to plague and the tissue smears and cultures may present bipolar staining organisms. A detailed account of these cases will be the subject of another paper.

The second class consists of findings from two, possibly three rat cadavers that were conclusively proven to be infected with B. tularense. Since no report of such an infection is available in the literature a brief account of the observations appears justified.

CASE REPORTS

Rat 1.—The animal had been trapped a few blocks from the central part of the city. A post mortem examination revealed lesions in some respects similar to those of plague. The subcutaneous tissues were slightly injected, the spleen was large, swollen and contained a few irregular whitish areas; the liver was definitely enlarged, dark in color and studded with numerous irregular necrotic foci varying in size from pin head to that of pin point. The pleural cavities contained a considerable amount of straw colored fluid although the lungs were free from macroscopic lesions. An examination of stained smears from the spleen and liver failed to reveal microorganisms.

The following staining solution has given uniformly good results: Methylene blue 2.5 Gm., basic fuchsin 0.15 Gm., carbolic acid 10 Gm., alcohol absolute 20 cc., water 200 cc. Stain is left in contact with the smear for one minute.

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Two guinea-pigs were inoculated with pieces of spleen and liver; guinea-pig A received the tissues subcutaneously (pocket method); in guinea-pig B the material was rubbed on the shaven skin (cutaneous vaccination).

Guinea-pig A died on the ninth day after inoculation and there was found general subcutaneous injection and marked gelatinous edema; fairly large right and left inguinal buboes; the right axillary nodes, both superficial and deep, definitely enlarged and hemorrhagic; spleen slightly enlarged and studded with necrotic areas varying from pin point to pin head size; liver enlarged and containing a number of irregular, small yellowish areas; lungs scarlet colored and edematous; both the peritoneal

TABLE 1

GUINEA-PIG INOCULATIONS

Guinea-pig	Type of Inoculation	Material Used	Day of Death After Inoculation
A	Subcutaneous	Liver and spleen of rat	9
B	Cutaneous	Liver and spleen of rat	Remained well
C	Subcutaneous	Liver and spleen of guinea-pig A	6
D	Cutaneous	Liver and spleen of guinea-pig A	8
E	Subcutaneous	Spleen of guinea-pig C	7
F	Subcutaneous	Spleen of guinea-uig D	7
G	Cutaneous	Spleen of guinea-pig D	10
H	Subcutaneous	Spleen of guinea-pig E	2 (died of pneumonia)
I	Subcutaneous	Spleen of guinea-pig F	11
J	Cutaneous	Spleen of guinea-pig F	8
K	Subcutaneous	Liver and spleen of guinea-pig G	10
L	Cutaneous	Liver and spleen of guinea-pig G	10
M	Cutaneous	Spleen of guinea-pig J	7
N	Cueaneous	Spleen of guinea-pig I	7
O	Cutaneous	Lung of guinea-pig I	13
P	Cutaneous	Spleen of culture guinea-pig 2	7
Q	Cutaneous	Spleen of culture guinea-pig 2	10

and pleural cavities filled with a straw colored fluid. Microscopic examinations of the tissues failed to reveal microorganisms. Agar plates and slants seeded with spleen, liver, and lymph-node fragments remained sterile even on prolonged incubation. Guinea-pig C received subcutaneously, and guinea-pig D cutaneously, spleen and bubo specimens from guinea-pig A. Guinea-pig B when chloroformed on the 12th day after inoculation was found free from gross lesions.

In a series of successive inoculations the infectious substance of rat 1, recovered from guinea-pig A, was passed through guinea-pigs C to Q. These inoculations were primarily made in order to preserve the organism until cultures were secured and furthermore to make observations on any possible increase in virulence. The data dealing with these experiments are summarized in table 1.

The course of the disease was subacute in 50% of the inoculated animals. As a rule the necropsy findings were nearly identical with those of guinea-pig A, namely subcutaneous injection and edema; enlarged hemorrhagic lymph nodes, occasionally containing yellowish, necrotic areas surrounded by a gelatinous exudate, enlarged spleen, and livers regularly permeated by yellowish, irregular, necrotic areas. Some animals revealed numerous pin point hemorrhages in the edematous lung parenchyma. Smears prepared from the organs showed a few minute bacilli, resembling cocci, some single, some in small clusters, all surrounded by a clear zone. In a number of instances cultures prepared from the spleen, liver, lungs and lymph nodes on egg yolk, diluted to 20% with 0.85% salt solution and coagulated [1] were successful. Once a supply of the special medium was available no difficulty was encountered in obtaining a fairly abundant growth after five to six days' incubation from all but one of the infected animals. Microscopically the growth

TABLE 2

RESULTS OF CUTANEOUS INOCULATION OF GUINEA-PIGS WITH CULTURES

Culture from Guinea-Pig	Generation in Vitro	Day of Death after Inoculation	Culture from Guinea-Pig	Generation in Vitro	Day of Death after Inoculation
D	1	7	J	1	8
D	1	8	M	1	7
E	1	14	O	1	11
F	1	5	Q	1	8
H	1	8	D	3	9
I	1	7	F	3	10

was composed of minute coccoid bacilli arranged in larger or smaller aggregations and seemingly enveloped by a capsular substance. Cutaneous inoculations were made with first and third generation cultures. The results of these experiments are shown in table 2.

It is evident from the data that guinea-pigs inoculated by the cutaneous route with pure cultures of an organism resembling in every respect B. tularense succumb in from 7 to 14 days to an infection which at autopsy presents the characteristic lesions already described.

One large ground squirrel, one wild rat (Mus decumanus s. norwegicus) and two white rats were treated cutaneously with tissue material secured from a guinea-pig which had died from the infection. One white rat died on the 10th day after the inoculation. At necropsy the cadaver showed a moderate, general, subcutaneous injection, enlarged

[1] McCoy, G. W., and Chapin, Ch. W., Pub. Health Bull., No. 53, 1912.

inguinal lymph nodes, an enlarged liver and spleen with several confluent necrotic patches and numerous hemorrhages in the moist lung parenchyma. It is worthy of note that the bacterioscopic examination of the organs demonstrated many clusters and single forms of B. tularense. The ground squirrel and the surviving wild and white rat were chloroformed on the 18th day. The rats presented no abnormalities; however, the axillary and pelvic lymph nodes of the ground squirrel were slightly enlarged and the liver as well as the spleen exhibited a few yellowish foci. No bacteria could be demonstrated in the stained smears. Unfortunately no guinea-pigs were inoculated with the tissues of these animals.

Transplants on egg yolk medium made from the original cultures grew abundantly after two to three days' incubation. The cystine-serum-glucose agar recommended by Francis [2] was very satisfactory for subcultures, although primary cultures directly from animal tissue could never be secured on this medium.

Through the courtesy of Dr. Edward Francis of the U. S. Public Health Service we were supplied with a specific agglutinating serum for B. tularense. All the cultures at our disposal were clumped by this serum in a dilution of 1 : 1,280. Control series with antiplague serum or with other organisms and with the tularense serum were uniformly negative.

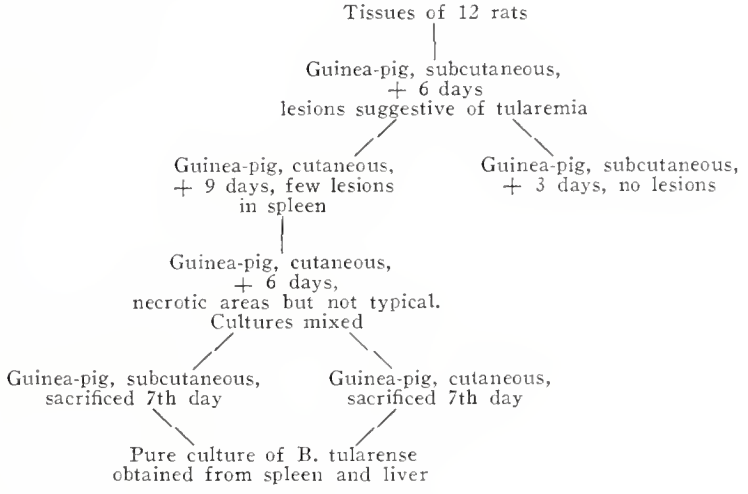
Rat 2.—The animal had been caught about six squares from the first and also in the central part of the city. The postmortem examination recorded an enlarged right inguinal lymph node, a dark and slightly enlarged liver with a few irregular yellow-whitish areas. The lung contained a few minute pin point hemorrhages; the spleen was normal in size and appearance. Microscopically no bacteria were demonstrated.

The guinea-pigs inoculated with material from the lymph nodes and liver died on the 8th day and exhibited at necropsy the typical lesions of infection with B. tularense, which was proven by cultural and serological tests.

Third Case.—A guinea-pig which had received a mass inoculation of the ground tissues of twelve rats showed at necropsy lesions suspicious for tularemia. The rats which furnished the material had been trapped in a section of the city approximately one to two miles removed from that in which rats 1 and 2 had been caught. Subinoculations were made

[2] Pub. Health Rep., 1922, 37, p. 987.

with the liver and spleen tissues of the infected guinea-pigs through a series of animals. For clearness the experiments are summarized.



The observations recorded leave no doubt that the production of lesions typical for tularemia in guinea-pigs and the purification of the strain requires repeated animal passages. Experience has taught that it is impossible to secure B. tularense in a pure state by plating tissues contaminated with other organisms. As already stated a pure strain gives all the identity reactions for B. tularense. The three strains have been maintained in guinea-pigs by vaccination for a period of from four to ten months, and although the time necessary to kill guinea-pigs has varied between six and twelve days the lesions have remained remarkably constant. In the subacute cases caseation of the inguinal and axillary lymph nodes as well as in the lungs is very marked, while in the acute stages the picture of scattered hemorrhages predominate. Characteristic lesions in the spleen and liver are invariably secured in guinea-pigs injected subcutaneously with pure cultures.

SUPPLEMENTARY DATA

Preliminary work has been undertaken with rats, both wild and tame in order to determine, if possible, the period during which this rodent may carry virulent B. tularense without showing symptoms or lesions of the disease. Although it is too early to give a complete report, some facts of considerable interest have been recorded. About 65% of the rats inoculated with a constant dose of a virulent culture of B. tularense succumbed to the infection. Macroscopically small hemorrhages

imbedded in a gelatinous exudate are the only visible lesions found at necropsy. Guinea-pigs inoculated with lung, liver and spleen tissue of these rats died with typical lesions of tularemia. The rats, which survived, were sacrificed at intervals varying from 10 to 21 days. None of the animals showed lesions suggestive of B. tularense infection but by inoculation experiment on guinea-pigs or by cultures the persistence of the bacteria in the spleen and liver was established. The heart blood was always found sterile.

Intraperitoneal injections of rats with cultures leads to an infection which is fatal in 48 hours. A general and marked injection of the subcutaneous tissues is the only autopsy finding.

Since tularemia is common among ground squirrels in the northern part of the state, particular attention has been paid to the rodents which were caught in the vicinity of Los Angeles. Mass inoculations of several thousand squirrels have been made but thus far no case of tularemia has been recognized.

SUMMARY

The existence of latent B. tularense infections in wild rats has been established by inoculation experiments, by cultures on egg yolk medium and by agglutination tests. The lesions in the naturally infected rats are not characteristic and the nature of the infection can only be established by inoculations of guinea-pigs.

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